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THE DETERMINATION OF URINARY ACID PHOSPHATASES[1]

BY G. E. DELORY AND MERLE HETHERINGTON

Abstract

The effect of dilution on the apparent acid phosphatase activity of undialyzed and dialyzed urine has been studied. In the former case, the apparent activity increases with dilution but this anomaly is removed by a preliminary dialysis. A convenient method for the determination of acid phosphatase based on this observation is described.

Introduction

In the application of standard methods for the determination of plasma and tissue phosphatase to urine, consideration must be given to the presence therein of interfering substances such as inorganic phosphate. Scott and Huggins (3) measured the amount of phosphate enzymically liberated from phenol phosphate by the urine after preliminary dialysis, but Burgen (1) tried to avoid this dialysis by using a method which measured the liberated phenol with diazotized *p*-nitroaniline. This seemed at first to be satisfactory but it was later (2) shown that misleading values could be obtained since the apparent phosphatase activity of the 24 hr. sample of urine increased as the urine was diluted.

Before beginning our studies with urinary acid phosphatases, it seemed important to investigate this problem from first principles to establish the cause of the anomaly and to ensure that those methods which involved a preliminary dialysis did not also exhibit the reported anomaly.

Since it seemed possible that the increase in apparent phosphatase activity might be due to the presence of inhibitory substances whose effect became less pronounced as the urine was diluted, it was decided to attempt to remove this inhibitor by dialysis. The success of the procedure was then assessed by comparing the effect of dilution on the apparent phosphatase activity of the dialyzed with that of the undialyzed urine.

Preliminary experiments led us to decide upon a method in which the amount of phosphate enzymically liberated from phenyl phosphate with an acetate buffer of pH 5.2 was measured.

[1] *Manuscript received July 16, 1951.*

Contribution from the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

Experimental

Procedure for the Measurement of Urinary Phosphatase Activity

A measured sample of urine was dialyzed against running tap water for three hours, after which the dialyzing thimble was transferred to a large beaker of distilled water and allowed to remain there overnight. This and an undialyzed sample were then analyzed as follows, allowance being made for dilution which had occurred during dialysis.

Into each of two centrifuge tubes were pipetted 2.0 ml. of $M/10$ disodium phenyl phosphate, and 2.0 ml. of acetate buffer pH 5.2. The tubes were placed in a water bath at 37° C. and allowed to reach that temperature. One milliliter of urine was then added, with mixing, to one of the tubes, the other serving as a blank. After 30 min., enzymic activity was stopped by the addition of 1.0 ml. of 10% trichloroacetic acid solution, which was also added to the blank followed by 1.0 ml. of the urine.

Each preparation was mixed and filtered. In each case 2.0 ml. of the filtrate, and 2.0 ml. of a standard phosphate solution, were treated with 1.0 ml. of acid ammonium molybdate solution and 0.2 ml. of aminonaphthol sulphonic acid solution. Water was added to a total volume of 10.0 ml., or more if the color intensity was high. Fifteen minutes at room temperature was allowed for maximum development of the color, which was then read in a photoelectric colorimeter with a red filter.

The unit of phosphatase activity was taken as the milligram of inorganic phosphate liberated in 30 min. by 100 ml. of urine under the conditions described.

The Effect of Dialysis and of Dilution upon Urinary Acid Phosphatase Activity

In this study, 24 hr. samples of urine were collected from an unselected group of normal people and hospital patients so that the procedure could be tested under varying conditions. The results of some typical experiments are shown in Table I. They show that with undialyzed urine there is usually an increase in apparent acid phosphatase activity which progresses with dilution, but that this anomaly is removed by dialysis. The most likely explanation of this is that urine contains a dialyzable inhibitor or inhibitors, the effect of which is lessened with increasing dilution. While no attempt was made to identify these substances, it seems probable that the effect is a composite one in which calcium, phosphate, and magnesium ions play a leading role.

Conclusion

The results presented here show that a preliminary dialysis is essential if consistent values for the urine acid phosphatase are to be obtained. Such a dialysis serves two purposes, it removes inhibitory substances and enables low blanks to be obtained when the enzymically liberated phosphate is to be measured.

TABLE I

EFFECT OF DILUTION ON THE APPARENT ACID PHOSPHATASE ACTIVITY OF DIALYZED AND UNDIALYZED URINE

Sample	Sex		Undiluted	Diluted 1 in 2	Diluted 1 in 10
1	F	Undialyzed	8.9	12.3	11.5
		Dialyzed	16.5	16.1	16.5
2	F	Undialyzed	19.6	25.0	49.5
		Dialyzed	21.9	21.4	21.8
3	F	Undialyzed	22.5	30.2	63.0
		Dialyzed	28.8	29.7	27.0
4	M	Undialyzed	121.8	143.4	181.8
		Dialyzed	111.1	110.7	108.1
5	M	Undialyzed	63.4	67.6	57.3
		Dialyzed	63.0	62.3	60.6
6	F	Undialyzed	16.0	14.9	37.3
		Dialyzed	27.2	27.8	26.4
7	F	Undialyzed	33.9	48.1	55.3
		Dialyzed	44.9	44.6	45.6
8	M	Undialyzed	34.9	42.1	50.4
		Dialyzed	42.6	40.7	42.6
9	M	Undialyzed	131.5	231.1	318.0
		Dialyzed	250.8	256.8	252.0

NOTE: *Figures represent units of phosphates per 24 hr. sample.*

Acknowledgments

Grateful thanks are due to Dr. F. D. White for his interest and encouragement, and to the National Cancer Institute of Canada for a grant in support of the project.

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THE ACID PHOSPHATASES OF HUMAN URINE[1]

By G. E. Delory and Merle Hetherington

Abstract

As part of an attempt to develop methods of estimating specifically the various acid phosphatases of the body, the inhibitory effect of 0.5% formalin and *M*/10 tartaric acid on the urine acid phosphatase activity of a group of healthy young men and women was studied. In respect to behavior to these inhibitors, the acid phosphatase of the urine from both sexes was similar to that of the seminal fluid and quite unlike that of the enzymes of the spleen, kidney, liver, and red cell.

Introduction

It has been known for many years that human urine possesses acid phosphatase activity (4) and there is indirect evidence (2, 6) that, in the male, it is derived principally from the prostate. It seemed to us therefore that a study of the urine acid phosphatases might prove profitable, expecially in view of the value of the determination of the plasma enzyme in the diagnosis and treatment of prostatic cancer. Such a study would be even more valuable if it were possible to determine the individual acid phosphatases separately. In an attempt to develop such methods, some unexpected findings were obtained which seem worthy of report.

Tissues known to possess acid phosphatase activity include not only the prostate but also the liver, kidney, spleen, and red blood cells. Since Abul Fad'l and King (1) have shown that formalin and *dl*-tartaric acid (which act as inhibitors) are useful in distinguishing between the enzymes of various origin, it was decided to attempt to confirm their findings and to apply them to the present problem. For the same purpose the effect of a number of metal ions was also investigated.

Experiments were carried out in which the effects of formalin and tartaric acid were studied both on dialyzed urine and on the preparations from other sources. Finally to check the validity of the assumption that the tissue enzymes would be unaffected by the presence of other substances in the urine, the behavior of a mixture of urine and another enzyme preparation was also studied.

Although alcohol has also been used (5) for the differentiation of serum acid phosphatases it was not utilized in the present study because of the dependence of its effect on salt concentration.

Methods and Materials

Determination of Acid Phosphatase Activity

Acid phosphatase activity was determined by the method of Delory and Hetherington (3). Substances to be tested for their action on the phosphatases

[1] *Manuscript received September 25, 1951.*

Contribution from the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

were added to the buffer and substrate mixtures before the addition of the enzyme so as to give the stated concentration in the hydrolysis mixture. Control experiments were carried out by substituting an equivalent volume of water for the substance under test.

Collection of Urine

Twenty-four hour samples of urine were collected from normal people and convalescent hospital patients. In every case, specimens were only collected from those persons whose intelligent cooperation could be relied upon.

The samples were collected into a bottle to which toluene had been added and the determination began as soon as practicable after the end of the 24 hr. period. Before the analysis was begun, the urine was dialyzed to remove inhibitory substances.

Other Phosphatase Preparations

Specimens of liver, kidney, and spleen were obtained from the autopsy room as soon as possible after death and extracted in a Waring Blendor with 20 times their weight of water, the time being kept to a minimum to avoid inactivation by denaturation. After the addition of a few drops of chloroform and toluene, the extracts were allowed to stand at room temperature for two days. At the end of this time, they were filtered and preserved in the ice-chest for use and diluted as required to give a suitable degree of activity.

Red cell preparations were obtained by centrifuging blood, removing the plasma and buffy layer, and washing the cells twice with isotonic sodium chloride solution. The washed cells were then haemolyzed with water to give a volume corresponding to a 1 in 100 dilution of the original blood sample.

Seminal fluid was diluted (usually 1 in 2000) to give a suitable activity.

Results

The Effect of Ions

The effect of certain metallic ions on urinary acid phosphatase activity is shown in Table I. All the ions studied exhibited some degree of inhibition, zinc being the most highly active.

TABLE I

INHIBITORY EFFECT OF CERTAIN IONS (CONC. $M/10$) ON THE ACID PHOSPHATASE ACTIVITY OF DIALYZED URINE

Sample	Sex	Degree of inactivation expressed as a percentage of the original activity					
		Mn	Ca	Mg	Co	Ni	Zn
1	F	13	9	12	15	8	63
2	F	23	11	14	22	10	63
3	M	11	8	9	11	8	58
4	M	15	9	9	13	8	63
5	F	25	11	18	24	9	69
6	F	19	19	17	13	4	66
7	M	11	8	9	16	15	64

Since, however, preliminary experiments and also the results of Abul Fad'l and King have shown that similar effects are obtained with red cell and liver enzymes, these studies were not pursued because of their limited usefulness as a means of differentiation.

The Effect of Formalin and Tartaric Acid

Table II shows the effect of formalin and of tartaric acid on the acid phosphatases of liver, kidney, spleen, red cells, and seminal fluid. These results are in general agreement with those of Abul Fad'l and King, although the

TABLE II

THE EFFECT OF (*A*) 0.5% FORMALIN AND (*B*) M/10 *dl*-TARTARIC ACID ON THE ACID PHOSPHATASE ACTIVITY OF VARIOUS TISSUES

Tissue	*A*			*B*		
	Untreated	Treated with formalin	% Inactivation	Untreated	Treated with tartaric acid	% Inactivation
Liver	8.4	5.5	34	8.4	2.6	69
	4.4	3.0	33	5.4	1.6	65
	7.8	5.5	29	7.8	2.5	68
Spleen	2.3	1.3	41	4.2	2.1	50
	2.2	1.3	39	3.8	1.5	61
	2.0	1.3	33	2.3	1.2	49
Kidney	10.1	4.2	58	4.4	1.8	58
	6.7	4.6	32	3.7	1.5	59
	2.8	1.9	32	6.7	2.2	67
Washed red cells	23.4	0.3	99	4.7	4.2	10
	25.4	0.7	97	10.2	10.0	3
	27.1	0.4	99	10.9	10.1	9
	31.1	0.4	99			
	32.7	0.2	98			
Seminal fluid	9.0	8.6	4	32.2	1.0	97
	8.1	7.4	8	15.3	0.6	96
	6.4	5.9	8	6.5	0.2	97
	6.5	6.1	6	12.4	0.4	97

NOTE: *Figures represent units of phosphatase per 100 ml. of original solution.*

inhibitory effect of formalin on seminal fluid under our conditions is a little less than was observed in their experiments. The most striking observation shown in these tables is that formalin has little effect on the seminal fluid phosphatase but strongly inhibits that of the red cell, while tartaric acid has the opposite effect in each case. In these respects, the red cell and seminal fluid acid phosphatases stand out in marked contrast to the other phosphatase preparations studied.

The effects of formalin and of tartaric acid on the urines of healthy young adults are shown in Tables III and IV respectively. It is to be noted that

TABLE III

EFFECT OF 0.5% FORMALIN ON URINARY PHOSPHATASE

Sample	Males			Sample	Females		
	Without formalin	With formalin	% Inactivation		Without formalin	With formalin	% Inactivation
M.L.S.	161	156	3.1	J.M.	46	43	6
G.W.	160	154	3.7	E.P.	61	56	8
J.T.	415	379	8.6	G.S.	41	39	4.4
J.G.	206	193	6.7	B.W.	80	74	7.5
J.S.	423	420	1	Y.D.	28	26	5.4
M.S.	295	284	3.7	B.D.	53	53	0
J.W.	296	288	2.7	J.F.	71	67	6.2
L.S.	184	179	2.5	J.F.	97	94	3
A.S.	240	232	3.2	C.B.	15	12	11
S.S.	113	105	6.8				
S.G.	128	121	5.5				
B.J.	512	480	6.2				
L.A.	204	195	4.4				

NOTE: *Figures represent phosphatase activity of 24-hr. samples taken from a normal group of ages 20–30.*

TABLE IV

EFFECT OF M/10 TARTARIC ACID ON URINARY PHOSPHATASES

Sample	Males			Sample	Females		
	Without tartaric acid	With tartaric acid	% Inactivation		Without tartaric acid	With tartaric acid	% Inactivation
S.C.	201	6	97	V.K.	41	1	97
M.M.	503	20	96	J.M.	71	2	96
R.C.	378	15	96	M.F.	80	2	97
W.D.	568	18	97	P.W.	59	2	96
C.B.	421	13	97	I.N.	35	0.4	99
T.C.	246	4	98	M.E.	50	1	98
B.M.	362	7	98				
R.B.	224	9	97				
P.C.	348	11	97				
G.H.	105	4	96				
G.P.	104	4	97				
W.P.	414	14	97				
L.A.	204	6	97				

NOTE: *Figures represent phosphatase activity of 24 hr. samples taken from a normal group of ages 20–30.*

the values obtained for total phosphatase (that is to say the phosphatase activity in the absence of added inhibitor) are greater in the males than the corresponding values in the females. This is not unexpected since it is believed that the main source of the urinary phosphatase in the male comes from the

prostate, although the lowest figure found for the males is (GP, 104) not very much higher than the highest for the females (JF, 97). These tables also show that the effect of formalin and of tartaric acid on the urinary acid phosphatase is very similar for both sexes, while comparison with Table II shows that this behavior is surprisingly very similar to the effect on the seminal fluid phosphatase and quite unlike that on the other phosphatase preparations studied.

In order to exclude the possibility that the acid phosphatases that we were studying were bacterial in origin, some samples of urine were examined which had been collected by catheterization and shown to be sterile; these samples behaved in exactly the same way to formalin and tartaric acid.

Finally, Table V presents the results of experiments carried out to determine whether our findings were due to other substances in the urine which were interfering with the action of the inhibitors used.

The results shown in this table prove this fear to be unjustified, since the values obtained by adding the various tissue preparations in turn to the urine are equal (within experimental error) to the sum of those of the urine and the other phosphatase preparation determined separately.

TABLE V

EFFECT OF ADDING DIALYZED URINE TO VARIOUS ACID PHOSPHATASE PREPARATIONS BEFORE AND AFTER TREATMENT WITH FORMALIN OR TARTARIC ACID

Tissue phosphatase	*A* Dialyzed urine + equal volume of water	*B* Tissue phosphatase and equal volume of water	*C* Equal parts of dialyzed urine and tissue phosphatase	Calculated value = (*A* + *B*)
Untreated				
Dialyzed kidney	4.7	2.8	7.7	7.5
Dialyzed spleen	2.7	0.5	3.6	3.2
Dialyzed liver	2.6	2.0	5.0	4.6
Dialyzed seminal fluid	11.6	17.3	29.2	28.9
Washed red cells	7.93	4.3	12.18	12.23
Treated with formalin				
Dialyzed kidney	2.4	1.6	4.4	4.3
Dialyzed spleen	2.6	0.6	3.2	3.2
Dialyzed liver	2.0	1.7	4.1	3.7
Dialyzed seminal fluid	1.9	6.5	8.4	8.4
Washed red cells	1.06	0.06	1.17	1.12
Treated with tartaric acid				
Dialyzed kidney	0.18	0.80	0.97	0.98
Dialyzed spleen	0.08	0.09	0.18	0.17
Dialyzed liver	0.04	0.09	0.17	0.13
Dialyzed seminal fluid	0.2	0.3	0.5	0.5
Washed red cells	0.06	4.37	4.21	4.43

NOTE: *The figures represent units of phosphatase activity per 100 ml. of sample.*

Discussion

The results presented have shown that the acid phosphatase of the urine from a group of young adults of both sexes is similar (irrespective of sex) in its behavior with formalin and tartaric acid to that of the seminal fluid. In the case of the urine of the young men this was not unexpected since it is widely held that the male enzyme is derived principally from the prostate but it was surprising to find that the urine of young women was also similar.

If the phosphatase of female urine were derived from the liver, red cells, spleen, or kidney, then one would expect this to be reflected in the effect of tartaric acid and formalin since, as has been shown, these substances affect the prostatic enzyme differently from those of other sources.

It appears therefore that human female urine contains an acid phosphatase (whose source has still to be identified) which is similar to that of the male prostate gland in the way in which it is affected by formalin and tartaric acid.

Acknowledgments

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CHANGES INDUCED IN THE ADRENAL CORTEX OF THE RAT BY THE WALKER RAT TUMOR 256[1]

By B. N. Kropp and H. D. McEwen

Abstract

The adrenal cortex in a series of 10 Wistar strain rats, inoculated with the Walker rat tumor 256, was studied histologically. Three types of cells, absent in normal controls, were found: (*a*) Cells containing coarse eosinophilic granules. These cells were few in number and confined to the zona glomerulosa. (*b*) Cells having the form of cortical cells, but with markedly basophilic cytoplasm, present in the zona fasciculata, often in dense masses and showing frequent mitoses. (*c*) Multinucleate giant cells present in the zona fasciculata. None of these cell types were seen in the adrenal medulla, kidney, liver, or spleen of these tumor-bearing rats. The possibility is advanced that the second cell type is derived from cortical cells, while the first and perhaps the third also are of myeloid origin. Neither the presence of these cells nor their abundance is related to tumor size.

Although the Walker transplantable rat tumor 256 is not known to metastasize extensively, induced changes in the adrenal glands, liver, and spleen, suggesting dysfunction of these organs, have been ascribed to its influence (1, 2, 3, 5, 6). Most of the adrenal cortical changes studied have been physiological, but some observations dealing with histological changes in the rat and mouse adrenal cortex have been described (1, 4, 6, 7, 8). Bearing most directly on the present study are the findings of McEuen and Selye (8); they found in the adrenals of rats, when the tumor weighed 50 gm. or more, areas of infiltrating cells in both medulla and cortex consisting of leucocytes and lymphocytes. In the cortex, in addition to these cells, others similar to adrenal cortical cells except for a basophilic cytoplasm were recognized. The infiltrating areas were found also in the liver, spleen, and periadrenal fat of these animals and were absent from normal ones. The condition was regarded as due to tissue decomposition originating within the tumor mass. No changes in the parenchymal cells were described. Lewis (7) similarly found in tumor-bearing rat adrenals areas of infiltration consisting of myeloid cells and found this condition in association with severe neutrophilia of the peripheral blood and myeloid hyperplasia of spleen, bone marrow, and certain lymph nodes.

Preliminary observations of organs from tumor-bearing rats showed histological pictures that frequently varied markedly from those previously described and led to the present study.

Materials and Methods

The animals used were 10 Wistar strain rats inoculated subcutaneously in the groin with the Walker rat tumor 256. The animals weighed 80 to 120 gm. at inoculation. They were sacrificed by decapitation, the tumors dissected

[1]*Manuscript received October 4, 1951.*

Contribution from the Departments of Anatomy and Pharmacology, Queen's University Medical School, Kingston, Ont. This investigation was aided by a grant from the National Cancer Institute of Canada.

TABLE I

CELL TYPES IN THE ADRENAL CORTEX OF TUMOR-BEARING AND CONTROL RATS

Animal No.	Tumor weight, gm.	Cell types in cortex		
		Eosinophilic	Basophilic	Giant cells
Tumor-bearing				
1	45	0	+	+
2	58	+	+	0
3	69	+	+++	+
4	71	+	+	0
5	84	+	++	+
6	87	0	+	+
7	90	+	+	0
8	91	0	+++	+
9	107	+	+	0
10	121	+	++	++
Controls				
1		0	0	0
2		0	0	0
3		0	0	0
4		+	0	0
5		0	0	0
6		0	0	0

NOTE: *The table was made up from the study of 10 complete sections through the center of the right adrenal gland of each animal. The symbols indicate the relative abundance of each cell type. 0 = absent; + = 1 to 12 cells counted in each section; ++ = 13 to 50 cells present; +++ = more than 50 cells present.*

out and found to weigh from 45 to 121 gm. (Table I). Animal No. 1 was sacrificed 19 days after inoculation, Animals 2 and 4 to 9 inclusive at 27 days, and Animals 3 and 10 at 29 days. The adrenal glands, as well as slices of kidney, liver, and spleen, were fixed in Bouin's fluid, and paraffin sections were stained in haematoxylin and eosin, or in iron haematoxylin. Serial sections through the entire right adrenal gland were taken, while representative sections of the other organs were cut and stained. Controls were a series of six uninoculated rats of the same strain which in all other respects were treated like the tumor-bearing animals.

Observations

Prominent histological changes were present in the adrenal cortex of all experimental animals, to a less extent or not at all in the medulla. Cortical capillaries and sinusoids always showed dilatation (9) with clogging of these vessels by erythrocytes and frequently by massed lymphocytes (Figs. 1, 6). Leucocytes, often immature forms, occluded some of the smaller vessels, but we did not find leucoyctes in the large numbers mentioned by others (7, 8), although some eosinophilic myelocytes were to be seen scattered through the loose connective tissue outside the capsule of the gland (Fig. 3). Lymphocytes, however, were present both in the dilated vessels and in dense masses

that displaced cortical cells, especially in the zona fasciculata. Aside from these cells, three principal cell types were seen in all our tumor-bearing rats:

(*a*) Ovoid cells, about 12 μ in diameter, with round or irregular nuclei, never lobed, and laden with coarse eosinophilic granules (Fig. 4). These cells were few in number and were not observed to form compact masses. They were seen isolated in the zona glomerulosa and were not found in the zona fasciculata, zona reticularis, or medulla.

(*b*) Polyhedral cells with strongly basophilic cytoplasm (Fig. 5). These cells were sometimes found scattered and isolated in all parts of the cortex but usually were present massed in the zona fasciculata. In most cells the nuclei were indistinguishable from those of cortical cells, except that in some instances it took on an elongated form. A nucleolus was present. In general these cells had the characteristic size and form of cortical cells. The basophilic cytoplasm, however, showed no obvious inclusions, nor did it show the typical vacuolated appearance of cortical cells. Among such cell clusters, or adjacent to them, masses of lymphocytes were occasionally present. No cells clearly of this type were seen massed in the adrenal medulla although an occasional isolated medullary cell could be interpreted as of the same type. Very striking were the large numbers of mitoses encountered in these massed basophilic cells. While obvious myeloid cells were also seen in division, so many of the dividing cells were of the basophilic type that there seemed no doubt of the ability of these latter cells to proliferate extensively.

(*c*) Multinucleate giant cells were present in small numbers (Fig. 6). These were 20 μ to 30 μ in diameter, basophilic, and frequently with vacuolated cytoplasm. The nuclei had a variety of forms; ring-shaped and polymorphous ones were most common, although several disconnected nuclei within a cell were also to be seen. Usually the nuceli seemed to be made up of the irregular coalescence of several nuclei, and changes in the basophilic cells suggestive of their transformation into giant cells could be followed in some instances. The giant cells were not seen in the medulla. Such large multinucleate cells were not distinguishable from the megakaryocytes of the spleen, and except for this fact, none of the three cell types described were ever encountered in the kidney, liver, or spleen of our tumor-bearing rats.

FIG. 1. Adrenal cortex. Rat No. 3, tumor weight 69 gm. Haematoxylin and eosin. × 40. The very dark staining masses in the outer cortex are infiltrating lymphocytes, the somewhat lighter masses basophilic cells.

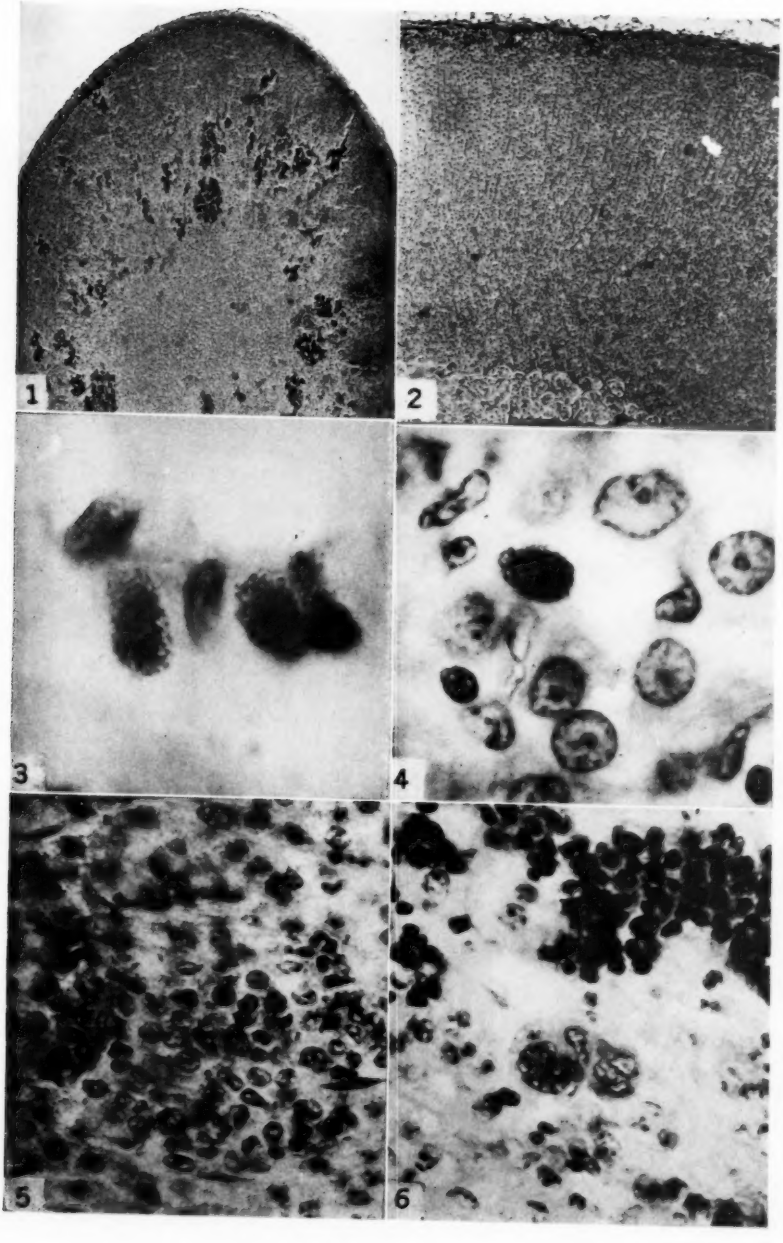
FIG. 2. Adrenal cortex. Rat No. 6, control. Haematoxylin and eosin. × 75.

FIG. 3. Cells with coarse eosinophilic granules in periadrenal connective tissue. Rat No. 10, tumor weight 121 gm. Haematoxylin and eosin. × 1200.

FIG. 4. Cell similar to those of Fig. 3, possibly an eosinophilic myelocyte, in zona glomerulosa. Rat No. 10, tumor weight 121 gm. Haematoxylin and eosin. × 1200.

FIG. 5. Basophilic cells mass in zona fasciculata. Rat No. 10, tumor weight 121 gm. Iron haematoxylin. × 650.

FIG. 6. Multinucleate giant cells in zona fasciculata, and mass of infiltrating lymphocytes. Rat No. 10, tumor weight 121 gm. Iron haematoxylin. × 750.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Discussion

Comparison of our findings with previous ones (7, 8) indicate great lability in the histology of the cortex of rats bearing the Walker tumor. Within the limits of the tumor sizes studied, the relative abundance of each cell type is not related to tumor size (Table I). Regarding the nature and possible origins of the three cell types described, the first may indeed be an eosinophilic myelocyte especially in view of the myeloid hyperplasia which has been described for these animals (7). If present in large numbers they may account for the areas described by McEuen and Selye (8) as of exclusively eosinophilic infiltration. Yet we failed to find them either in comparable numbers or in a location other than the outermost portion of the cortex and the adjacent connective tissue (Figs. 3, 4). The basophilic cells may be identical with the basophilic epithelioid cells mentioned by the same authors. It is interesting, however, that while these have been found in small numbers previously, they were the most abundant type in our specimens. The possibility is present that they represent a form of haemocytoblast, and this might account for the presence and formation *in situ* of the megakaryocyte-like cells. Yet the structural similarity between the basophilic cells and the cortical cells leaves open the further possibility that the former represent a stage in the breakdown of cortical cells under the influence of the necrotizing tumor.

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THE EFFECT OF AGE, SEX, AND PREVIOUS PREGNANCY ON THE OXYGEN UPTAKE OF RAT MAMMARY TISSUE[1]

By Jules Tuba and M. Shirley Fraser

Abstract

The effect of age, sex, and breeding on oxygen consumption and response to *p*-phenylenediamine of normal rat mammary tissue has been studied. Oxygen consumption values (Q_{O_2}) are significantly higher for breeder females than for virgin females or males. The endogenous oxygen consumption by breast tissue of virgin rats more closely resembles that of males than of breeder females. There is a significant lowering of Q_{O_2} with age in all the animals. Oxygen consumption in the presence of *p*-phenylenediamine ($Q_F^{O_2}$) is not significantly different for mammary tissue of males and breeders of the youngest age group (12 – 25 weeks) but it is higher for virgins of the same age. A highly significant fall in $Q_F^{O_2}$ to approximately equal levels appears at a comparatively early age (30 – 50 weeks) in males and virgins, but this does not occur in the breeder female rats until the ability to reproduce has diminished or ceased (50 weeks and over). Reserves of the cytochrome system ($Q_F^{O_2} - Q_{O_2}$) are lower in breeders than in males or virgins and are decreased by age in the latter two groups. These studies indicate the importance of considering age, sex, and breeding history of animals used for mammary tissue respiratory studies.

Introduction

A previous report from this laboratory (2) described an *in vitro* investigation of oxygen consumption and response to *p*-phenylenediamine of adult rat mammary tissue. Females in a number of experimental states as well as normal males were used. It was observed that the response to *p*-phenylenediamine was almost identical for mammary tissue from normal male rats and nonlactating breeder females. However, reserves of the cytochrome system and endogenous oxygen consumption were significantly altered in mammary tissue by pregnancy and lactation. It therefore appeared that hormonal influences and the extent of glandular development affected the reserves of the iron-containing enzyme system as well as respiration. We decided to extend this earlier investigation to include the effect of age, sex, and previous pregnancy on the oxygen uptake and the response to *p*-phenylenediamine of normal rat mammary tissue. This essential information would then be available for subsequent studies of the variations which might occur in cytochrome system reserves in spontaneous or experimentally induced pathological conditions involving rat mammary glands.

Experimental

Male and female rats of the Wistar strain were used. The range of ages included animals from 12 to 68 weeks, which almost entirely covered the reproductive period of the females. The animals were housed in large cages, generally in groups of six, and were given water and Purina Fox Checkers ad libitum. Males and virgin females were kept segregated from the beginning

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta. The investigation was supported by a grant from the National Cancer Institute of Canada.

of the experiment. Breeder females were not sacrificed until at least four weeks after they had weaned their last litters, to ensure the mammary tissue being in the resting state.

Manometric methods were the same as those reported in our first paper (2). Inguinal glands were used in all cases. Before the glands were sliced the lymph nodes were removed since these are nonglandular. The values of the endogenous oxygen uptake (Q_{O_2}) for mammary tissue slices as well as oxygen uptake in the presence of *p*-phenylenediamine ($Q_P^{O_2}$) were calculated in μl. per mgm. per hr. on the basis of dry, fat-free weight. The importance of reporting oxygen consumption values on a fat-free basis, because of the very high fat content of rat mammary tissue (about 90%), was emphasized previously.

Results

The values of Q_{O_2} for mammary tissue of male, virgin female, and breeder female rats are arranged in three age groups in Table I. Statistically significant differences are indicated and in each case the value of "*t*" and the level of significance are given. There is no significant difference in the endogenous

TABLE I

OXYGEN CONSUMPTION (Q_{O_2}) OF MAMMARY TISSUE OF MALE, VIRGIN FEMALE, AND BREEDER FEMALE RATS

(Numerical values are averaged and standard errors of means are indicated. Q_{O_2} values are based on fat-free, dry weight of mammary tissue)

Age in weeks	Males		Virgins		Breeders	
	No. of rats	Q_{O_2}	No. of rats	Q_{O_2}	No. of rats	Q_{O_2}
12-25	10	3.4 ± 0.51	12	2.9 ± 0.26	6	4.0 ± 0.24
30-50	12	2.7 ± 0.21	18	2.9 ± 0.12	12	4.1 ± 0.44
Over 50	4	1.9 ± 0.21	3	2.2 ± 0.18	12	3.0 ± 0.13

Statistical analysis of data of Table I

Groups compared	Value of "*t*"	Value of "*P*"
12-25 week males vs. males over 50 weeks	2.68	< 0.02
30-50 week virgins vs. virgins over 50 weeks	3.33	< 0.01
30-50 week breeders vs. breeders over 50 weeks	2.39	< 0.05
30-50 week males vs. 30-50 week breeders	2.86	< 0.01
Males over 50 weeks vs. breeders over 50 weeks	4.44	< 0.01
12-25 week virgins vs. 12-25 week breeders	3.10	< 0.01
30-50 week virgins vs. 30-50 week breeders	2.63	< 0.02
Virgins over 50 weeks vs. breeders over 50 weeks	3.61	< 0.01

oxygen consumption of mammary tissue of males and virgin females in any age group. The Q_{O_2} for breeder females is significantly higher than for virgins and this significant difference persists for all ages. This suggests that hormonal factors and glandular development affect respiration of rat mammary tissue, and the observation is in agreement with our previous work (2). Q_{O_2} values for the male rats are also significantly lower than for the breeders, with the exception of the youngest age group. There is a significant lowering of Q_{O_2} in older males, virgins, and breeder females.

The data in Table II were obtained with the animals used for Q_{O_2} estimations reported in Table I. Males and breeder females of the youngest group showed no significant difference in $Q_P^{O_2}$ and this agrees with our previous observation

TABLE II

OXYGEN CONSUMPTION IN PRESENCE OF *p*-PHENYLENEDIAMINE ($Q_P^{O_2}$) OF MAMMARY TISSUE OF MALE, VIRGIN FEMALE, AND BREEDER FEMALE RATS

(Same animals as were used for Table I. Numerical values are averaged and standard errors of means are indicated. $Q_P^{O_2}$ values are based on fat-free, dry weight of mammary tissue)

Age, in weeks	Males, $Q_P^{O_2}$	Virgins, $Q_P^{O_2}$	Breeders, $Q_P^{O_2}$
12-25	7.4 ± 0.32	8.7 ± 0.56	6.9 ± 0.14
30-50	5.0 ± 0.34	5.6 ± 0.23	6.9 ± 0.37
Over 50	5.3 ± 0.38	5.4 ± 0.81	5.1 ± 0.26

Statistical analysis of data of Table II

Groups compared	Value of "*t*"	Value of "*P*"
12-25 week males vs. 30-50 week males	5.12	< 0.01
12-25 week virgins vs. 30-50 week virgins	5.00	< 0.01
30-50 week breeders vs. breeders over 50 weeks	4.00	< 0.01
12-25 week virgins vs. 12-25 week breeders	3.01	< 0.01
30-50 week virgins vs. 30-50 week breeders	2.98	< 0.01

(2). Virgins of the 12 – 25 week group have significantly higher levels of $Q_P^{O_2}$ than the breeders of the same age. Oxygen consumption, in the presence, of *p*-phenylenediamine, by mammary tissue of breeders remains constant until the animals are past the breeding age, when a highly significant decrease occurs. Significant decreases in $Q_P^{O_2}$ values appear at an earlier age in male and virgin female rats. In the 30 – 50 week group of virgins the lack of mammary development which is associated with previous pregnancy becomes strikingly reflected in the oxygen consumption. These values of $Q_P^{O_2}$ are significantly below those for breeder females and closely approximate the values for males.

According to Craig, Bassett, and Salter (1) the difference between $Q_P^{O_2}$ and Q_{O_2} is a measure of the cytochrome reserves of tissues. The differences for the animals used in this investigation, calculated from the data of Tables I and II, are given in Table III. The use of 1.85×10^{-2} *M* *p*-phenylenedi-

TABLE III

INCREASE IN OXYGEN CONSUMPTION CAUSED BY THE ADDITION OF *p*-PHENYLENEDIAMINE TO RAT MAMMARY TISSUE SLICES ($Q_P^{O_2} - Q_{O_2}$)

(Numerical values are averaged and standard errors of means are indicated)

Age in weeks	Males, $Q_P^{O_2}-Q_{O_2}$	Virgins, $Q_P^{O_2}-Q_{O_2}$	Breeders, $Q_P^{O_2}-Q_{O_2}$
12-25	4.0 ± 0.64	5.7 ± 0.55	2.9 ± 0.17
30-50	2.3 ± 0.30	2.7 ± 0.22	2.8 ± 0.31
Over 50	3.3 ± 0.32	3.1 ± 0.74	2.2 ± 0.21

Statistical analysis of data of Table III

Groups compared	Value of "*t*"	Value of "*P*"
12-25 week males vs. 30-50 week males	2.40	< 0.05
12-25 week virgins vs. 30-50 week virgins	5.09	< 0.01
12-25 week virgins vs. 12-25 week breeders	4.87	< 0.01

amine as a substrate indicates that there are, on a fat-free, dry-weight basis, appreciable reserves of cytochrome in rat mammary tissue. The values of $Q_P^{O_2} - Q_{O_2}$ are highest in the youngest group of males and virgins. The effects of age in males and virgins and of glandular development in breeding females are to lower the reserves to what appears to be a basic level of cytochrome.

The results in the tables indicate that oxygen consumption by male and virgin female rat mammary tissue follows a similar pattern. Mammary development in breeder female rats results in a pattern characterized by higher endogenous oxygen consumption during the reproductive part of life, as well as by lower cytochrome reserves.

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THE RELATIONSHIP OF DIETARY FACTORS TO RAT SERUM ALKALINE PHOSPHATASE

II. THE EFFECT OF TOTAL FOOD CONSUMPTION, METHIONINE, CHOLINE, AND VITAMIN B_{12} IN NORMAL AND ALLOXAN DIABETIC ADULT RATS[1]

By Jules Tuba and Neil B. Madsen

Abstract

Food consumption in alloxan diabetic rats shows a positive correlation with (1) alkaline phosphatase activity, (2) blood glucose level, (3) body weight, similar to the correlation between enzyme activity and food intake in normal adult rats when they are fed a stock laboratory diet. Oral vitamin B_{12} has no effect on serum alkaline phosphatase levels of normal or diabetic rats, nor does it modify the effect of supplementary methionine in either group. The effect of supplementary choline on the phosphatase of normal rats is unaltered by this vitamin which is reported to have transmethylating and lipotropic influences. Methionine added to animal checkers lowers phosphatase levels in both normal and diabetic adult rats, but this is directly related to lowered food consumption which always accompanies supplementation with this amino acid. The animals maintain their weights and appear to be in good condition, so it may be assumed that the lowered intake of food is adequate. Supplementary choline decreases phosphatase levels of normal rats by about 20% of initial values, and this appears to be due to choline itself and not to altered food consumption. Phosphatase levels of alloxan diabetic rats, however, are slightly but significantly elevated by supplementary choline.

Introduction

The first paper of this series (4) dealt with the effect of dietary fat, methionine and cystine on the serum alkaline phosphatase activity of weanling male albino rats. Variations in the concentrations of fat and methionine in the synthetic diets fed to growing rats produced marked alterations in the levels of the enzyme, as well as in the daily food consumption. Cantor, Wight, and Tuba (1) found that the abnormally high serum alkaline phosphatase levels associated with alloxan diabetic adult male rats could be influenced by variations in the diets received by the animals. This paper is concerned with the effect of supplementary methionine, already studied in weanling animals, on the serum alkaline phosphatase and food consumption of normal and alloxan diabetic adult male rats. Vitamin B_{12}, which is reported to have lipotropic and transmethylating effects (2), was used as a supplement as well as the lipotrope, choline.

Experimental

Diabetes was induced in adult male albino rats by the subcutaneous injection of alloxan. These and the normal male control animals were fed ad libitum a basal diet of ground Purina Fox Checkers or the basal diet supplemented as indicated below. Animals were housed in single cages and the weight and daily food consumption of each animal were noted at regular intervals during

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton Alta., with financial assistance from the National Research Council, Ottawa, Canada.

the period each diet was fed. At the same time serum alkaline phosphatase and blood sugar levels were determined by methods described in previous publications (3, 4).

Results

The Relationship Between Food Consumption and Serum Alkaline Phosphatase of Alloxan Diabetic Rats

Nine adult male rats which manifested well established alloxan diabetes were placed in individual cages and they were given ground Purina Fox Checkers and water ad libitum for three days. At the end of that time the daily food consumption, weights, blood glucose, and serum alkaline phosphatase levels were determined for each animal. They were then fed successive decrements of food as indicated in Table I for three day periods. At the end of each period, the animals were weighed and estimations were made of the blood glucose and serum phosphatase.

TABLE I

THE EFFECT OF LIMITING CONSUMPTION (GM. PER DAY) ON SERUM ALKALINE PHOSPHATASE LEVELS (UNITS PER 100 ML.); BLOOD GLUCOSE (MGM. %), AND BODY WEIGHTS (GM.) OF DIABETIC RATS. (MEANS OF NINE RATS)

Each food level was fed for three days before estimations were carried out

Consumption	26 ± 1** (100)*	24 (93)	19 (73)	14 (54)	9 (35)	4 ± 0** (15)
Phosphatase	335 ± 17** (100)	354 (105)	247 (73)	177 (53)	135 (40)	61 ± 5** (18)
Blood glucose	504 ± 44** (100)	429 (85)	295 (59)	262 (52)	244 (48)	135 ± 11** (27)
Body weight	218 ± 9** (100)	213 (98)	195 (89)	186 (85)	173 (79)	146 ± 8** (67)

* *Figures in brackets are percentages of zero values.*
** *Standard error of mean.*

The correlation of mean serum alkaline phosphatase activities with food consumption is 0.99. The formula of the regression line shown in Fig. 1 is $P = 13.27\ C + 6$, where P is the phosphatase activity in units per 100 ml. and C is the daily food consumption in grams. The regression coefficient has a t value of 13.27 ($P < 0.01$) and the correlation between food consumption and enzyme activity is highly significant.

Correlation of blood glucose levels and of body weights with food consumptions are also highly significant.

The Effect of Dietary Supplements Fed to Normal and Alloxan Diabetic Adult Rats

The effect on serum alkaline phosphatase of feeding various levels of food to normal adult male rats has been reported by Cantor, Wight, and Tuba (1)

and a highly significant correlation of 0.97 was obtained. Tuba, Baker, and Cantor (3) found that variations in this serum enzyme occurred in male and female rats following gonadectomy or injection of various sex hormones, and

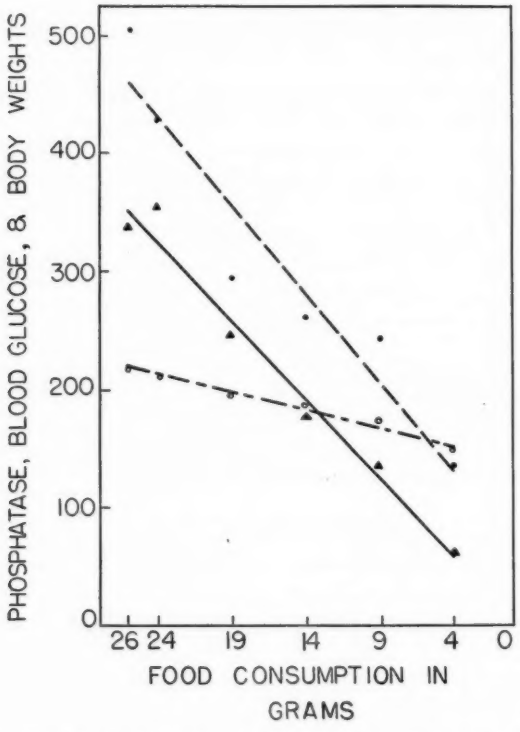

FIG. 1. The relationship between total food consumption and serum alkaline phosphatase (———), blood glucose (— —), body weights (— - — -).

that these changes could be related to food consumption. The correlation which in this instance was 0.70, was significant, but it could also be concluded that other factors might exert a modifying influence.

The concentration of dietary fat appears to be very closely associated with levels of alkaline phosphatase in rat serum. Statistical analysis of the results of Cantor, Wight, and Tuba (1) obtained with adult animals on limited, subnormal food intake, indicates a highly significant correlation of 0.86 ($P<0.01$) between the daily intake of fat and the serum enzyme. The work of Tuba and Shaw (4) with weanling rats fed various synthetic diets ad libitum, further confirms the relationship and in this case the correlation is 0.98 ($P<0.01$). It was noted from the results of Tuba and Shaw and from unpublished data from this laboratory that the influence of dietary fat may be

modified by other factors in the diet. When methionine is used as a supplement, the usual correlation between food consumption and enzyme activity is not observed, or in other words, supplementary methionine in itself produces a significant alteration in the serum alkaline phosphatase levels of normal weanling rats.

The action of methionine in lowering serum alkaline phosphatase levels, presumably because of the lipotropic effect of the labile methyl groups, led to the use of this amino acid as a dietary supplement in normal and alloxan diabetic adult rats. Another lipotropic substance, choline, was also used as a supplement as well as vitamin B_{12}, reported to have transmethylating and lipotropic actions (2). In all the following experiments a basal diet of ground Purina Fox Checkers was used.

The Effect of Vitamin B_{12}

Saline solutions of crystalline vitamin B_{12} were used. Oral supplements of this solution, equivalent to 1.0 μgm. daily, were given to eight normal and seven alloxan diabetic adult rats for three days. The result, not reported here, showed that supplementary vitamin B_{12} had no significant effect on serum alkaline phosphatase of normal or alloxan diabetic animals.

The Effect of Methionine Alone or With Vitamin B_{12}

(*a*) *Normal Rats*

A group of 10 adult male rats was fed ad libitum a diet of ground checkers to which methionine had been added to a concentration of 3%. Another group of 10 animals received the same diet but in addition were given oral supplements of 1.0 μgm. of vitamin B_{12} daily after the eighth day. The two groups were maintained for 17 days on their respective diets. The results in Table II give a correlation of serum alkaline phosphatase activity with food intake of 0.92 ($P<0.01$) for the group receiving supplementary methionine and of 0.90 for the group receiving supplements of methionine and vitamin B_{12}. Therefore in normal adult male rats the decrease in levels of the enzyme associated with methionine supplementation of the basal diet can be related to lowered food consumption. This is contrary to the findings with weanling animals reported by Tuba and Shaw. Vitamin B_{12} produced no significant effect on the enzyme in addition to that attributable to methionine.

(*b*) *Alloxan Diabetic Rats*

The preceding experiment was repeated with alloxan diabetic adult male rats for 21 days. The results in Table III give a correlation of 0.99 ($P<0.01$) between enzyme activity and food intake in the group receiving methionine and a correlation of 0.96 for the group receiving supplementary methionine and vitamin B_{12}. Therefore, in neither group did the supplementation of the diet affect the serum alkaline phosphatase levels, as in the experiment above with normal adult animals.

In all four groups of adult animals receiving supplementary methionine, after an initial adjustment to the dietary regime, weights remained constant in spite of decreased consumption. In previous experiments with weanling

TABLE II

THE EFFECT OF SUPPLEMENTS OF METHIONINE ALONE AND METHIONINE WITH VITAMIN B_{12} ON SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P); DAILY FOOD CONSUMPTION (GM.) (C); AND BODY WEIGHTS (GM.) (W) OF NORMAL RATS. (MEANS OF 10 ANIMALS ARE REPORTED IN EACH GROUP)

Vitamin B_{12} supplementation was begun on the eighth day

Days	Methionine			Methionine + B_{12}		
	P	C	W	P	C	W
0	119 ± 6** (100)*	15.6 ± 0.8** (100)	239 ± 9** (100)	113 ± 7** (100)	16.6 ± 0.4** (100)	235 ± 12** (100)
3	90 (76)	13.1 (84)	234 (98)	79 (70)	12.8 (77)	228 (97)
7	98 (83)	12.3 (79)	235 (98)	88 (78)	11.9 (72)	229 (98)
9	81 (68)	11.2 (72)	231 (97)	69 (61)	10.5 (63)	225 (96)
14	102 (86)	14.3 (92)	237 (99)	88 (78)	14.4 (87)	229 (98)
17	106 ± 7** (89)	15.0 ± 0.2** (96)	242 ± 8** (101)	94 ± 4** (83)	15.3 ± 0.1** (92)	235 ± 3** (100)

* *Figures in brackets are percentages of zero values.*
** *Standard error of the mean.*

rats fed moderate supplements of methionine the rate of growth was similar to that of animals receiving the unsupplemented diet, although consumptions were appreciably lower (4). It would appear that supplementation with methionine results in nutritionally adequate diets which are characterized by lowered consumption.

The Effect of Choline Alone or with Vitamin B_{12}

(*a*) *Normal Rats*

Ten male adult rats were fed a diet consisting of the basal diet containing a supplement of 1.5% choline and another group of 10 normal animals received the same diet and in addition oral supplements of 1.0 μgm. vitamin B_{12} each daily after the eighth day. Statistical analysis of the results in Table IV show that there is no correlation between food consumption and phosphatase activity in either group. The results further indicate that supplementary choline produces a lowering of about 20% in both groups. Comparison of mean phosphatase levels at the beginning of the experiment and at its termination 14 days later shows a significant decrease with a t value of 6.05 ($P<0.01$) in the first group. During the experiment the animals gained weight, which,

TABLE III

THE EFFECT OF SUPPLEMENTS OF METHIONINE ALONE AND METHIONINE WITH VITAMIN B_{12} ON SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P); DAILY FOOD CONSUMPTION (GM.) (C); BODY WEIGHTS (GM.) (W); AND BLOOD GLUCOSE (MGM. %) (G) OF DIABETIC RATS. (MEANS OF 10 ANIMALS ARE REPORTED IN EACH GROUP)

Vitamin B_{12} supplementation was begun on the eighth day. The control group was returned to the stock diet on the 21st day

Days	Methionine				Methionine and B_{12}			
	P	C	W	G	P	C	W	G
0	307 ± 20*** (100)**	27.5 ± 0.3*** (100)	226 ± 7*** (100)	532 ± 32*** (100)	334 ± 9*** (100)	26.6 ± 1.6*** (100)	206 ± 6*** (100)	531 ± 19*** (100)
7	213 (69)	19.5 (71)	210 (93)		217 (65)	19.6 (74)	184 (89)	
9	219 (71)	19.1 (70)	210 (93)		235 (70)	18.7 (70)	185 (90)	
14	209 (68)	18.3 (67)	210 (93)		235 (70)	18.5 (70)	189 (92)	
21	206 ± 14*** (67)	18.7 ± 0.7*** (68)	211 ± 16*** (93)	348 ± 12*** (65)	231 ± 10*** (69)	19.1 ± 1.8*** (72)	190 ± 8*** (92)	376 ± 16*** (71)
29*	271 ± 16*** (88)	24.6 ± 2.0*** (89)	235 ± 15*** (104)	454 ± 21*** (86)				

* *Eight days on stock diet.*

** *Figures in brackets are percentages of zero values.*

*** *Standard error of the mean.*

TABLE IV

THE EFFECT OF SUPPLEMENTS OF CHOLINE ALONE AND CHOLINE WITH VITAMIN B_{12} ON SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P); DAILY FOOD CONSUMPTION (GM.) (C); AND BODY WEIGHTS (GM.) (W) OF NORMAL RATS. (MEANS OF 10 ANIMALS ARE REPORTED IN EACH GROUP)

Vitamin B_{12} supplementation was begun on the eighth day

Days	Choline			Choline + B_{12}		
	P	C	W	P	C	W
0	150 ± 6** (100)*	18.2 ± 0.3** (100)	224 ± 5** (100)	130 ± 7** (100)	19.2 ± 0.4** (100)	224 ± 5** (100)
3	137 (91)	17.7 (98)	229 (102)	113 (87)	17.9 (93)	231 (103)
7	129 (86)	16.6 (91)	240 (107)	118 (91)	17.2 (90)	242 (108)
9	118 (78)	15.1 (83)	239 (107)	109 (84)	15.9 (83)	243 (109)
14	118 ± 7** (78)	17.0 ± 0.4** (94)	252 ± 20** (113)	106 ± 7** (82)	17.7 ± 0.3** (92)	248 ± 6** (111)

* *Figures in brackets are percentages of zero values.*

** *Standard error of the mean.*

in our experience, serves to emphasize the importance of the decrease in enzyme activity. Comparison of final phosphatase values for the two groups shows that vitamin B_{12} had in itself no effect on the enzyme.

(*b*) *Alloxan Diabetic Rats*

Ten animals which had been diabetic for three weeks were used. This period ensures the use of animals still in relatively good condition and with serum alkaline phosphatase activity leveled off at about 200–300% above normal (1). They were fed the basal diet containing a supplement of 1.5% choline for six weeks and at the end of that time the amount of added choline was increased to 2.5% for seven additional days. A slight but statistically significant increase was found in the phosphatase values, which shows no correlation with food consumption. A rise of similar magnitude was noted in an identical experiment.

The effect of supplementary choline on the abnormally elevated alkaline phosphatase activity of the serum of alloxan diabetic rats is the opposite of the findings with normal rats. This finding further emphasizes the metabolic abnormalities found in this type of experimental diabetes.

The effect of oral vitamin B_{12} on the serum phosphatase activity of alloxan diabetic animals receiving supplementary choline was not investigated.

TABLE V

THE EFFECT OF CHOLINE SUPPLEMENTATION ON SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P); DAILY FOOD CONSUMPTION (GM.) (C); AND BODY WEIGHTS (GM) (W) OF DIABETIC RATS. (MEANS OF 10 ANIMALS)

The choline supplement was increased to 2.5% at the end of six weeks

	P	C	W
0 Values	302 ± 10** (100)*	27.3 ± 0.9** (100)	212 ± 6** (100)
1 Week	350 (116)	28.1 (103)	207 (98)
2 Weeks	328 (109)	30.4 (111)	208 (98)
3 Weeks	335 (111)	27.8 (102)	206 (97)
4 Weeks	328 (109)	27.7 (101)	204 (96)
5 Weeks	331 (110)	26.3 (97)	204 (96)
6 Weeks	332 ± 6** (110)	26.8 ± 1.2** (98)	203 ± 7** (96)
1 Day on 2.5% choline	302 (100)	23.8 (87)	199 (94)
3 Days on 2.5% choline	332 (110)	25.7 (94)	200 (95)
7 Days on 2.5% choline	340 ± 7** (113)	24.2 ± 0.4** (89)	205 ± 8** (97)

* *Figures in brackets are percentages of zero values.*
** *Standard error of the mean.*

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ON RAT SERUM LIPASE

III. THE EFFECT OF TOTAL FOOD CONSUMPTION AND OF DIETARY HYDROGENATED VEGETABLE FAT[1]

By Jules Tuba and Jack D. Taylor

Abstract

There is no correlation in either weanling or adult normal male rats between daily food consumption of a stock laboratory diet and serum tributyrinase activity. Limiting the food intake of adult male rats has no statistically significant effects on serum tributyrinase. The abnormally elevated serum tributyrinase levels of alloxan diabetic adult male rats may be accounted for on the basis of increased food consumption. Weanling and adult male animals maintained on synthetic diets containing from 5 to 60% fat show a correlation between serum tributyrinase concentration and daily fat consumption. In the case of adult rats increased fat ingestion is accompanied by increased enzyme activity. However, with weanling rats increased fat consumption results in lowered tributyrinase concentrations and retarded growth. The results with growing animals are attributed to the decreased intake of food, and the corresponding decrease in protein consumption, which accompanies the increase in dietary fat concentration. Replacement of the synthetic diets by the stock laboratory diet is followed by altered tributyrinase levels in both weanling and adult animals.

Introduction

A striking characteristic of adult rat serum lipase noted by Tuba and Hoare (5) was that throughout a starvation period of one week enzyme concentrations remained at normal levels. Repetition of this experiment by us indicated that this finding held true for periods of starvation up to 14 days and only a day or so before death did lipase values decrease. It therefore appeared probable that variations in food intake would not likely account for the altered lipase levels which were found associated with various experimental states (5). Independence of serum lipase concentration and daily food consumption would thus be the opposite of findings in this laboratory with rat serum alkaline phosphatase. In the case of the latter enzyme, Tuba and Madsen (6) have shown a highly significant correlation with consumption of food, particularly of fat, except in the presence of certain modifying dietary factors. In spite of the findings with starved animals, an attempt was made to correlate serum lipase levels of both weanling and adult male rats with the daily ingestion of stock laboratory diet and of a hydrogenated vegetable fat, Crisco. The relationship of food consumption and activity of the enzyme was considered in alloxan diabetic adult male rats as well.

Experimental

Animals were housed in individual cages and were given food as described below for individual experiments. Weights were noted at weekly intervals and these observations were used as a check on the health or rate of growth

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial aid from the National Research Council, Ottawa, Canada.

of the animals on the different diets. Data concerning weights is included in the tables only when correlation with lipase concentrations has been found.

In order to determine the effect of total daily food consumption the animals were maintained on the stock laboratory diet of Purina Fox Checkers, which were powdered in order to minimize scattering by the rats. This was fed to weanling, normal adult, and alloxan diabetic adult rats. The effect of fat was tested by feeding synthetic diets which contained 5%, 10%, 25%, 40%, and 60% of this dietary constituent to weanling and normal adult male rats. Each synthetic diet contained 2% cod liver oil and the remainder of the fat was made up with Crisco, a hydrogenated vegetable fat. Each of this group of diets contained 20% casein, 40% McCollum's salt mixture, and the remaining fraction was made up with sucrose in every case. Adequate amounts of the following vitamins were added: thiamine hydrochloride, pyridoxine hydrochloride, calcium pantothenate, niacin, riboflavin, and choline. Consumption by each animal was noted daily. In order to simplify reports of data and statistical studies, consumption figures for each group were totaled every week, and the values appearing in the tables are the averages in grams per rat per day for each week of the test period for each diet.

Serum tributyrinase levels were determined once a week by means of the titrimetric micromethod of Tuba and Hoare (4). The tributyrinase activity of serum in units is equivalent to the number of milliliters of 0.025 *N* sodium hydroxide required to neutralize the butyric acid set free by the enzyme contained in 100 ml. serum in 30 min. at pH 8.05 and 37° C.

Results

The Effect of Total Food Consumption

In Normal Weanling and Adult Male Rats

Six weanling male rats, 21 days old, and six adult rats were placed on the diet of powdered Checkers, which they received ad libitum for six weeks. The means of the results for these two experiments are given in Table I. There is no correlation between daily food consumption and serum tributyrinase activity in either growing or mature animals ($r = 0.06$ and 0.01 respectively). There is, however, a highly significant correlation between the weights of the animals and tributyrinase levels in the weanling group ($r = 0.56$ at the 1% level). This further confirms the observations of Tuba and Hoare (5) that serum tributyrinase levels increase from low levels at birth to higher levels which become constant in the mature animal. The lack of correlation between the weights of the adult group and their serum tributyrinase levels ($r = 0.16$) is therefore to be expected.

The effect of limiting the food intake of normal adult animals was tested also. Six mature male rats which had been eating powdered Checkers ad libitum for several weeks were then limited to 15 gm. each daily for one week. During the following four weeks they were maintained on daily levels of 12, 9, 6, and 3 gm. of food for one week on each successive decrement. The mean serum tributyrinase levels for the group remained within normal limits for the

TABLE I

THE RELATIONSHIP BETWEEN DAILY FOOD CONSUMPTION AND SERUM LIPASE (TRIBUTYRINASE) LEVELS IN WEANLING AND ADULT MALE RATS RECEIVING A DIET OF POWDERED ANIMAL CHECKERS

(Averages for six animals in each group)

Time after beginning of experiment, weeks	Weanling rats			Adult rats	
	Consumption in gm./day/rat	Serum lipase, units/100 ml.	Weights in grams	Consumption in gm./day/rat	Serum lipase, units/100 ml.
1	12.0 ± 0.5*	—	73 ± 5*	22.0 ± 0.5*	760 ± 30*
2	15.0 ± 0.5	510 ± 30*	113 ± 6	22.0 ± 0.5	610 ± 40
3	18.0 ± 0.5	610 ± 10	156 ± 7	21.0 ± 0.5	560 ± 20
4	19.0 ± 0.5	590 ± 10	187 ± 5	22.0 ± 0.5	580 ± 10
5	20.5 ± 0.5	600 ± 20	228 ± 6	21.0 ± 0.5	680 ± 30
6	20.0 ± 0.5	610 ± 20	240 ± 5	20.0 ± 0.5	660 ± 40

* *Standard error of the mean.*

first four weeks of the experiment. Only at the end of the week on a daily intake of 3 gm. of food did the results given in Table II show a decline from normal mean serum lipase concentrations. Once more there is no significant

TABLE II

THE EFFECT OF LIMITING FOOD CONSUMPTION ON SERUM LIPASE (TRIBUTYRINASE) LEVELS OF ADULT MALE RATS

Each food level except the first was fed for one week
(Averages for six animals)

Consumption in gm./day/rat	Serum lipase, units/100 ml.
17 ± 0.5* (ad libitum)	610 ± 20*
15	520 ± 30
12	590 ± 20
9	670 ± 20
6	610 ± 30
3	350 ± 90**

* *Standard error of the mean.*

** *The mean serum tributyrinase value and the standard error of the mean are affected by subnormal levels of the enzyme in the sera of four animals (320, 290, 240, and 20 units per 100 ml.) which died a few hours after they were bled. The remaining two rats, which survived the final bleeding for lipase estimation, had tributyrinase levels within the normal range (700 and 510 units per 100 ml.).*

correlation between food consumption and serum tributyrinase levels. The value of r, which is at the extreme lower limit of the 5% level of significance, is 0.33. Weight and activity of the enzyme showed no correlation in this experiment.

In Alloxan Diabetic Adult Male Rats

Six alloxan diabetic rats with pronounced hyperglycemia were used for this experiment. The mean serum lipase activity of the group was well above normal, in accordance with the findings of Tuba and Hoare (5). These animals were fed powdered Checkers ad libitum for six days. The amount of food eaten by each animal was measured daily and serum lipase activity was estimated every two days. The mean lipase values for this group are given in Table III as well as the mean average food consumption for the 24 hr. immediately preceding the bleeding for determination of tributyrinase levels.

TABLE III

THE RELATIONSHIP BETWEEN DAILY FOOD CONSUMPTION AND SERUM LIPASE (TRIBUTYRINASE) LEVELS IN ALLOXAN DIABETIC ADULT MALE RATS

(Averages are for six animals in the group)

Time after beginning of experiment, days	Consumption in gm./day/rat	Serum lipase in units/100 ml.
2	32 ± 1.3*	800 ± 60*
4	35 ± 1.2	890 ± 100
6	33 ± 0.9	810 ± 100

* *Standard error of the mean.*

The regression line shown in Fig. 1 was calculated from the three lipase values and the six daily consumption levels determined for each rat during the course of the above experiment. The calculated equation for this regression line is $T = 44C - 636$ (T = tributyrinase and C = food consumption) and this has a highly significant t value = 3.1 ($P < 0.01$). This, together with the value of $r = 0.64$ for the data of Table III, indicates that there is a highly significant correlation between food ingested and serum tributyrinase concentration in alloxan diabetic adult male rats.

A simple arithmetic comparison of the consumption and enzyme data of the preceding three tables seems to indicate that the abnormally high tributyrinase activity associated with alloxan diabetes in rats is largely attributable to the increase in food consumption. This can readily be proved statistically. We have used the individual consumption and lipase data for six alloxan diabetic rats (Table III) and for six normal adult rats (last three weeks in Table I) and there is a highly significant correlation; $r = 0.63$ with a t value of 3.59 at the 1% level.

It was shown above that this correlation does not exist in normal adult rats receiving food ad libitum or in limited amounts. When the animals are starved or receive restricted amounts of food it would appear that the levels of serum tributyrinase must remain at or above certain unknown values and that the rats die when the enzyme concentration falls below these levels. The lack of correlation in normal adult rats eating unrestricted amounts of

powdered Checkers is probably due to the wide fluctuations in the activity of the enzyme in a group of animals eating much the same quantity of food from day to day and week to week (Table I), and it should be noted that the value

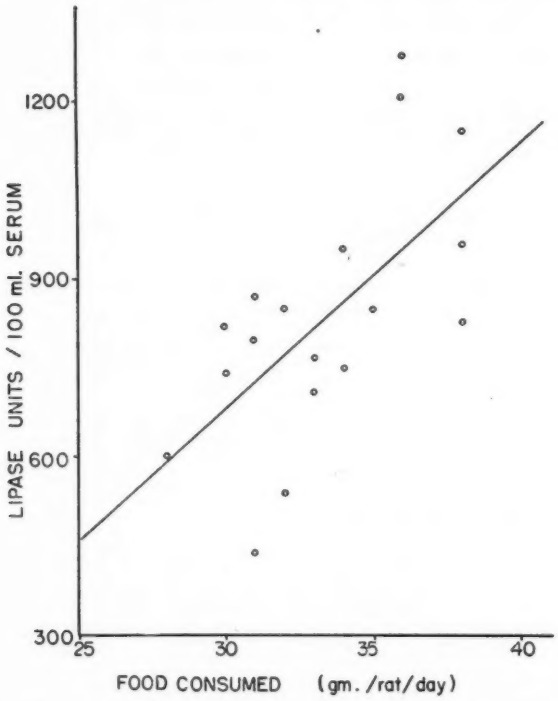

FIG. 1. The regression line for the relationship between daily consumption of a stock laboratory diet and serum tributyrinase levels in alloxan diabetic adult male rats.

of r for this experiment is almost significant. Only when both consumption and lipase vary markedly from the normal ranges, as in the alloxan diabetic group (Table III) is this correlation readily shown.

The fact that a correlation between food intake and serum tributyrinase concentration could be obtained under certain conditions led to a consideration of the effect on the enzyme of various dietary constituents, notably fat. In this regard, Hess and Viollier (3) have reported that the absence of fat from the diet reduces the serum tributyrinase content of rat plasma.

The Effect of Dietary Hydrogenated Vegetable Fat In Weanling Male Rats

Groups of 10 weanling male rats were fed ad libitum for six week periods on a number of synthetic diets containing the various levels of fat indicated in Table IV. Each diet contained 2% cod liver oil and the balance of the fat

TABLE IV

THE EFFECT OF DIETARY FAT CONCENTRATION ON MEAN DAILY FOOD CONSUMPTION (GM. PER DAY) (C), MEAN DAILY FAT CONSUMPTION (GM. PER DAY) (F), AND SERUM TRIBUTYRINASE (UNITS PER 100 ML.) (T) OF WEANLING MALE RATS

(Averages are for 10 animals in each group)

Fat in diet, %	2 weeks			3 weeks			4 weeks			5 weeks			6 weeks		
	C	F	T	C	F	T	C	F	T	C	F	T	C	F	T
5	11.0	0.55	530	10.8	0.54	550	11.4	0.57	540	14.1	0.70	570	14.1	0.70	530
10	8.2	0.82	550	9.1	0.91	500	12.0	1.20	470	12.3	1.23	470	10.0	1.00	470
25	10.2	2.53	510	10.2	2.53	480	13.5	3.38	530	10.2	2.53	450	12.0	3.00	410
40	6.0	2.40	380	7.8	3.12	440	7.5	3.00	370	12.4	4.97	450	14.0	5.52	330
60	6.1	3.96	400	7.0	4.20	430	8.3	4.98	370	7.6	4.56	400	6.2	3.72	300

was made up with Crisco. Lipases were estimated weekly except at the end of the first week, when the animals were too small to bleed. The daily food intake of individual animals was variable on some of the diets, especially when higher concentrations of fat were fed. Consequently the values for food consumption reported in Table IV in grams eaten per rat per day are the means of the total food eaten by the 10 animals of each group over a period of one week. From the food consumption data the amount of fat eaten per rat per day has been calculated.

A significant correlation exists between the amount of fat eaten daily by weanling rats and the levels of serum tributyrinase. The value of $r = 0.71$. The formula of the regression line shown in Fig. 2 is $T = -33F + 540$ (T = tributyrinase and F = fat consumption) and this has a highly significant value of $t = 5.2$ at the 1% level. The standard error of estimate for the line in $\pm$ 51.

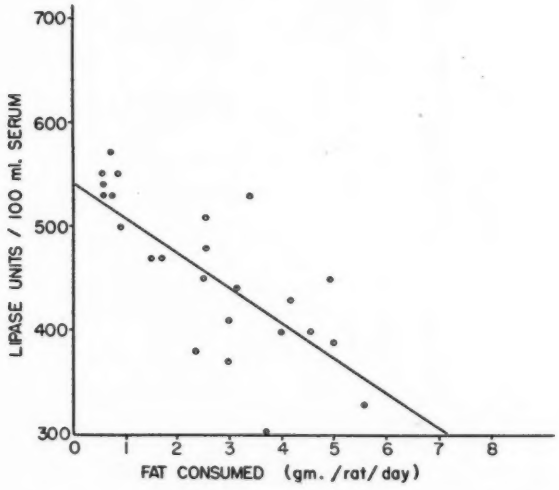

FIG. 2. The regression line for the relationship between serum tributyrinase levels and fat consumed per day by normal weanling rats.

In Adult Male Rats

The preceding experiment was repeated with a group of 10 normal adult male rats. Lipases were determined at the end of each week of the feeding period. As with the weanling animals the average daily consumption of food became less as the fat concentration of the diets was increased. The daily consumption of fat once more was higher as the dietary fat concentration was increased. A highly significant correlation exists between the consumption and enzyme data given in Table V ($r = 0.78$ at the 1% level).

TABLE V

THE EFFECT OF DIETARY FAT CONCENTRATION ON MEAN DAILY FOOD CONSUMPTION (GM. PER DAY) (C), MEAN DAILY FAT CONSUMPTION (GM. PER DAY) (F), AND SERUM TRIBUTYRINASE (UNITS PER 100 ML.) (T) OF ADULT MALE RATS

(Averages are for 10 animals in each group)

Fat in diet, %	1 week			2 weeks			3 weeks			4 weeks			5 weeks			6 weeks		
	C	F	T	C	F	T	C	F	T	C	F	T	C	F	T	C	F	T
5	14.1	0.71	400	13.6	0.68	410	11.4	0.57	440	12.0	0.60	470	14.6	0.73	420	15.0	0.75	450
10	14.7	1.47	410	13.0	1.30	370	12.0	1.20	470	15.0	1.50	420	14.8	1.48	400	14.6	1.46	380
25	15.2	3.80	520	12.6	3.15	520	11.8	2.95	540	11.4	2.85	430	11.3	2.82	440	11.2	2.80	440
40	11.4	4.56	570	11.8	4.72	650	8.8	3.52	660	11.8	4.72	590	12.3	4.92	640	12.0	4.80	660
60	13.6	8.16	590	9.1	5.45	590	8.4	5.04	550	10.4	6.25	500	9.5	5.70	540	9.7	5.83	550

The formula of the regression line shown in Fig. 3 is T = + 33F + 385 (T = tributyrinase and F = fat consumption) and this has a highly significant t value of 6.9 at the 1% level. The standard error of estimate for this regression line is ± 55.

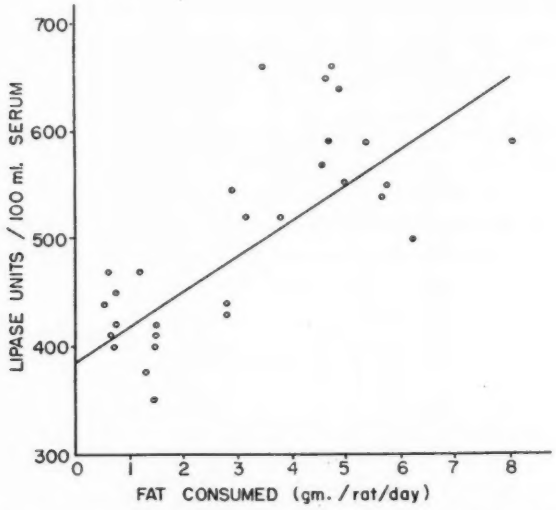

FIG. 3. The regression line for the relationship between serum tributyrinase levels and fat consumed per day by normal adult rats.

A striking fact concerning the regression lines for the above two experiments is that the slopes have opposite signs. The increased lipolytic activity of serum associated with increased fat ingestion in the case of adult animals could be expected if serum tributyrinase has a role in the metabolism of fats. The findings with weanling animals is surprising. The data in Table VI offers some explanation.

TABLE VI

THE RELATIONSHIP BETWEEN AVERAGE GROWTH RATE OF WEANLING MALE RATS (IN GM.) ON VARIOUS DIETARY CONCENTRATIONS OF FAT AND AVERAGE SERUM TRIBUTYRINASE ACTIVITY (T) (UNITS PER 100 ML.)

Diet	Weight after 6 weeks on diet	Weight gain in 6 weeks	T at end of 6 weeks	Weight gain after one week on Checkers	T after one week on Checkers	Change in T produced by Checkers
Checkers, 5% fat	240	185	610	30	—	—
Synthetic, 5% "	213	142	570	40	610	+ 40
" 10% "	201	149	470	7	550	+ 80
" 25% "	180	121	410	31	660	+250
" 40% "	183	138	330	30	560	+230
" 60% "	147	92	300	41	420	+120

The growth of weanling rats fed the various diets listed in Table VI is best on Checkers, which contain about 5% fat. Growth on the synthetic diets containing 5 and 10% fat is not quite as good, but they are not as palatable as the Checkers, judging by consumption data. The diminished rate of growth on all the synthetic diets can be accounted for on the basis of lowered daily food consumption which in nine-week-old rats (at the end of the six week feeding periods) ranges from 20 gm. on the Checker diet to 6.2 gm. on the 60% fat diet. The synthetic diets which contain more than 5% fat not only interfere with the rate of growth, because of inadequate protein intake, but tend to maintain the mean lipase levels near the values of 317–484 units per 100 ml. reported by Tuba and Hoare (5) for three- to four-week-old rats.

After the termination of the feeding experiments, each group of animals was placed on Checkers for one week. An improvement in the rate of growth was noted in all but one group, and the enzyme levels increased toward the range of values characteristic of the stock laboratory diet.

Deuel and his co-workers (2) found that rats fed ad libitum on synthetic diets containing 5 to 50% fat showed better growth than those fed lower concentrations of fat or a stock laboratory diet. Their results were similar for cottonseed oil and a hydrogenated vegetable fat, margarine. Barki, Collins, Elvehjem, and Hart (1) fed various levels of three vegetable oils and butter to growing rats and observed that the dietary concentration of each for optimal growth was characteristic of the fat. These levels were 10% for corn oil and 35% for butter fat, whereas coconut oil or soybean oil concentrations had no effect on growth. In our experiments, which were primarily designed for lipase studies, the closest approximation to both "normal" growth and serum tributyrinase activity was obtained with the synthetic diet containing 5% fat. Our results are probably affected to some extent by the fact that each synthetic diet contained 2% cod liver oil. However, experiments are now in progress with this and a number of other fats to see what effect they have on growth and lipase concentration.

The adult animals used in our six week feeding tests gained 110 gm. on Checkers and from 85 to 125 gm. on the various synthetic diets. Transference from the synthetic diets to a diet of powdered Checkers for one week resulted in a return of tributyrinase activity to levels ranging from 510 to 630 units per 100 ml. which are typical for the stock laboratory diet.

The problem of constancy of tributyrinase levels during starvation remains unanswered. Since diet does affect the activity of the enzyme, one can only assume until further work is done, that the starvation levels are associated with utilization of body stores of fat.

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THE EFFECT OF PROPYLTHIOURACIL ON THE WEIGHT AND CONCENTRATIONS OF CHOLESTEROL AND ASCORBIC ACID OF THE ADRENAL GLAND[1]

By William F. Perry

Abstract

The effect on the rat adrenal gland of massive doses of propylthiouracil over a three-week period has been studied. Care was taken to have any body weight changes of the treated animals paralleled by similar changes in the control animals. Under these circumstances it was found that on a gland weight basis no atrophy of the adrenal glands occurred. There were however alterations in the adrenal cholesterol and ascorbic acid concentrations, there being an increase in the former and a decrease in the latter and further these changes were not dependent upon any hypometabolism induced by the thiouracil. The level of the adrenal cholesterol and ascorbic acid concentrations measured three hours after injection of ACTH and of epinephrine indicated that the adrenal cortex was responsive to its trophic hormone and that the pituitary–adrenal axis was intact, the degree of response being similar in both treated and control animals.

Several investigators (1, 6, 7, 8, 14) have reported that atrophy of the adrenal cortex can be brought about by the administration of thiouracil or related compounds. Usually the experimental animals have been young growing rats given moderate doses of thiouracil (0.1-0.5% in drinking water or diet) for periods of time ranging from 10 to 100 or more days. Although histological evidence of cortical atrophy as a result of such treatment has been produced (1, 2) and in at least one instance paired feeding of control animals has been done (7), most workers have used as evidence of atrophy a decline in adrenal weight compared with the gland weight of untreated animals allowed to eat ad libitum.

The following work reports the effect of three weeks' treatment with massive doses of thiouracil on the weight and certain chemical constituents of the adrenal cortex of young adult rats. As thiouracil leads to a decline in body weight due in part to a lowered food consumption which of itself might cause a loss of adrenal weight or alteration in function, it seemed advisable to adjust the food intake of the control animals so as to have parallel weight changes with the treated animals.

Methods

Young adult male albino rats of a highly inbred strain weighing between 160 and 200 gm. were used as experimental animals. The animals were handled in pairs whose initial weights did not differ by more than 12 gm.; one of each pair was given 80 mgm. propylthiouracil suspended in 4 ml. water by stomach tube daily for 21 days. The other member of each pair served as a

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Contribution from the Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Canada.

control. The animals' food intakes and weights were recorded daily and so adjusted that any body weight changes of the treated animals were paralleled by similar changes in the controls.

On this regime a few of the treated animals died before the 21 days of the experiment had elapsed; their controls were also removed from the series. After 21 days the animals were killed by means of an overdose of nembutal given intraperitoneally, the adrenal and thyroid glands quickly removed and weighed on a torsion balance. In most cases the left adrenals were then analyzed for their cholesterol content by the Sperry–Schoenheimer method (11) and the right adrenals for the ascorbic acid content by the method of Roe and Oesterling (12). In still other animals treated for 21 days the response of the adrenal cholesterol and ascorbic acid concentrations to injections of either 5 mgm. ACTH (Armour) or 0.3 ml. 1/1000 epinephrine, given three hours previously, was measured and the results obtained compared with those obtained on the control animals treated in a similar manner.

The above measurements were also carried out on groups of rats given the same daily dose of propylthiouracil but in addition were also given daily injections of 10 μgm. *l*-thyroxine. These animals were compared with control and thiouracil treated animals. In each of these groups of three, food intake was adjusted so that the weight changes varied with those of the animals given only the goitrogen.

Results

It was noted that immediately following the institution of the thiouracil treatment the animals' food intake declined markedly, leading to considerable loss of weight. This state continued for the first 7 to 10 days of the experiment, following which the animals commenced to increase their food intake gradually and by the end of the experimental period of 21 days they had regained their initial weights to a considerable extent.

In Table I is shown the results on 22 rats following three weeks' treatment with 80 mgm. of propylthiouracil daily. It will be seen that, compared with those of the pair fed control animals, the adrenal glands of the treated animals showed no change in weight, irrespective of whether absolute or proportional

TABLE I

THE EFFECT OF PROPYLTHIOURACIL ON ADRENAL WEIGHTS

Animals	Initial wt., gm.	Final wt., gm.	Adrenal wt.	
			Mgm.	Mgm./100 gm.
22 Controls	177	178	27.3 ± 1.1*	15.1 ± 0.6*
22 Treated	168	164	25.9 ± 1.1	15.9 ± 0.8

* = ± *S.E.*
P = > .05.

adrenal weights are considered. It would appear that on the basis of gland weight large doses of thiouracil do not bring about any atrophy of the adrenal.

The effect of the treatment on the concentration of cholesterol and ascorbic acid of the glands is shown in Table II. There were changes in both these constituents. The cholesterol concentration was very significantly increased

TABLE II

THE EFFECT OF PROPYLTHIOURACIL ON THE ADRENAL CHOLESTEROL AND ASCORBIC ACID CONCENTRATIONS

Group	Animals	Adrenal cholesterol, mgm./100 mgm. gland ± S.E.	Adrenal ascorbic acid, μgm./100 mgm. gland ± S.E.
1	20 Controls	4.53 ± 0.26	451 ± 21
2	20 Propylthiouracil	6.06 ± 0.52	386 ± 26
3	15 Propylthiouracil and thyroxine	5.98 ± 0.27	409 ± 40

NOTE:

$$P \begin{cases} \textit{Group 1 vs. Group 2} = < .01 \\ \textit{Group 2 vs. Group 3} = > .05 \\ \textit{Group 1 vs. Group 3} = < .01 \end{cases} \quad \textit{(cholesterol concentrations)}.$$

$$P \begin{cases} \textit{Group 1 vs. Group 2} = < .05 \\ \textit{Group 2 vs. Group 3} = > .05 \\ \textit{Group 1 vs. Group 3} = > .05 \end{cases} \quad \textit{(ascorbic acid concentrations)}$$

while that of the ascorbic acid was depressed to a significant degree when compared with the concentrations of the control animals. Also shown in this table are the cholesterol and ascorbic acid concentrations following three weeks treatment with 80 mgm. daily of propylthiouracil plus 10 μgm. daily of *l*-thyroxine. This amount of thyroxine is generally considered to be equivalent at least to the daily requirement of the rat for thyroid hormone (3, 9). It will be seen that the adrenal cholesterol concentration was unaffected by such treatment, being still significantly higher than the control values; the mean ascorbic acid concentration although apparently somewhat restored toward the control level did not differ significantly from the concentration in the glands of animals receiving only thiouracil.

That the doses of thyroxine given in conjunction with the thiouracil adequately overcame the hypothyroidism induced by the thiouracil is indicated by the results shown in Table III. It will be seen that the goitrogenic action of the thiouracil, and hence presumably the hypothyroidism, is abolished by such doses. In addition thyroxine alone led to a decline in thyroid weight compared to the control thyroid weights suggesting the dosage used was at least equivalent in activity to the daily requirement of thyroid hormone by the animals.

In Table IV is shown the ascorbic acid and cholesterol concentrations of control, propylthiouracil treated, and propylthiouracil and thyroxine treated

TABLE III

THE EFFECT OF PROPYLTHIOURACIL AND THYROXINE ON THYROID WEIGHT

Animals	Thyroid, mgm./100 gm.	
	Mean	Range
22 Controls	9.5	7.3 – 13.2
22 Propylthiouracil	28.3	18.7 – 49.8
18 Propylthiouracil and thyroxine	9.6	6.1 – 11.6
16 Thyroxine	5.3	3.8 – 7.4

TABLE IV

THE EFFECT OF ACTH ON ADRENAL CHOLESTEROL AND ASCORBIC ACID

No.	ACTH dose, mgm.	Cholesterol, mgm./100 mgm. gland	% Decline	Ascorbic acid, μgm./100 mgm. gland	% Decline
20 Controls	0	4.53	—	451	—
10 Controls	5	3.41	25	302	33
20 Propylthiouracil	0	6.06	—	386	—
9 Propylthiouracil	5	4.50	25	295	24
15 Propylthiouracil and thyroxine	0	5.98	—	409	—
5 Propylthiouracil and thyroxine	5	3.86	35	235	42

TABLE V

THE EFFECT OF EPINEPHRINE ON THE ADRENAL CHOLESTEROL AND ASCORBIC ACID

No.	Epinephrine, ml.	Cholesterol, mgm./100 mgm.	% Decline	Ascorbic acid, μgm./100 mgm.	% Decline
20 Controls	0	4.53	—	451	—
18 Controls	0.3	2.60	43	252	44
20 Propylthiouracil	—	6.06	—	386	—
12 Propylthiouracil	0.3	3.32	45	258	34
15 Propylthiouracil and thyroxine	—	5.98	—	409	—
8 Propylthiouracil and thyroxine	0.3	3.44	42	214	47

rats three hours after subcutaneous injections of ACTH. There were similar percentile declines in both cholesterol and ascorbic acid in all groups of animals indicating that the thiouracil treated gland is responsive to its trophic hormone.

Similar results were obtained three hours after the injection of 0.3 ml. of 1/1000 epinephrine. The results of this experiment which are set forth in Table V indicate that the animals' pituitaries are able to liberate adequate amounts of ACTH in response to an alarming stimulus.

Discussion

The work reported above fails to show that any adrenal atrophy, as judged on a weight basis, occurs as a consequence of daily administration of heavy doses of thiouracil. In addition the functional capacity of the glands, as revealed by changes in cholesterol and ascorbic acid content after ACTH and/or epinephrine, is likewise unaffected by the propylthiouracil treatment.

However, it has been shown that thiouracil brings about certain chemical changes in the adrenal, namely an increase in the cholesterol concentration together with a decreased ascorbic acid concentration and further that these changes are independent of any hypometabolism or athyroidal state which might occur as a consequence of thiouracil administration. It would appear that these changes are due to some more direct action of the drug on the adrenal cortex.

There are a number of reports (1, 6, 7, 8, 14) claiming that thiouracil causes adrenal atrophy, i.e. a decline in weight as compared with control animals' organs, either on an absolute weight basis or expressed as a ratio of organ to body weight. In most of these experiments it does not appear that body weight changes of control and treated animals were kept similar as was the case in the experiments reported here. That this is of importance may be seen in the work of Williams *et al.* (13) who demonstrated that thiouracil leads to a general growth retardation and weight loss of all organs. Leathem (7) who used pair fed controls in his experiments reported that despite a loss of body weight on thiouracil the ratio of adrenal to body weight was depressed after 20 days of thiouracil and concluded that there was a true cortical atrophy. However his pair fed control animals gained considerably more weight over the experimental period than did the treated animals.

Cytological studies by Deane and Greep (2) showed that there was an involution of the gland confined to the zone fasciculata; identical histological results were obtained following thyroidectomy and they concluded that any thiouracil induced atrophy was merely a result of hypothyroidism. Baumann and Marine (1) reported that all zones of the cortex shrank following thiouracil but there was a higher content of lipoid staining material. This latter finding agrees with our observation of an increase in cholesterol content. This might be interpreted as adrenals in a resting state (10) and if so one would expect the ascorbic acid concentration to be elevated also (10). As has been shown here and by others (4, 5) the ascorbic acid concentration is lower than normal. Gabrilove and Soffer (5) interpret this low concentration of ascorbic acid as consistent with a depression of cortical function but a lowered ascorbic acid content has also been shown to be characteristic of an active gland (10).

The present work confirms the findings of Zarrow and Zarrow (15) who showed that the glands of thiouracil treated rats were as responsive to exogeneous ACTH as those of normal animals; these workers also reported a weight loss in the adrenal and suggested that the increased secretion of thyrotrophin by thiouracil treated rats was at the expense of the pituitary ACTH secretion. Both the present report and that of Gabrilove and Soffer (5) show that the adrenals of thiouracil treated rats as well as being responsive to exogeneous ACTH are also responsive to epinephrine, indicating no defect in pituitary production or release of ACTH.

In conclusion it may be stated that, using gland weight as a basis (provided body weight changes of control and treated animals are kept similar), it does not appear that thiouracil induces any adrenal atrophy or that there is any defect in the ability of the cortex to respond to exogenous or endogenous ACTH. There is, however, a change in the cholesterol and ascorbic acid concentrations, there being an increase in the former and a decrease in the latter. Furthermore, these changes are independent of any hypometabolic state resulting from thiouracil administration and it is suggested that they are due to some more direct action of the drug on the adrenal cortex. The interpretation of these alterations in the chemical constituents of the adrenal gland remains to be clarified.

Acknowledgments

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RADON IN NORMAL BREATH[1]

By E. J. Martin and J. K. W. Ferguson

Abstract

When radon-free air is breathed prior to sampling, the amounts of radon in the expired air of normal subjects are considerably less than the amounts commonly found in "fresh" atmospheric air. The radon content of expired air of 15 normal men after breathing radon-free air for 10 min. was found to be 1.5×10^{-14} curies per liter, with a standard error of 0.24×10^{-14} curies. The origin of radon in normal breath is discussed.

Introduction

Under normal conditions the human body contains a certain amount of the radioactive gas, radon, which has been absorbed as such from the surrounding air and other sources, or has been generated from the radium in the body. Being a chemically inert gas, radon is distributed through the body fluids and tissues in concentrations determined largely by its solubility coefficients. Its distribution between aqueous liquids and gases is such that at equilibrium the concentration in the gaseous phase is the higher one. Consequently expired air is more suitable for analytical studies than blood or urine (9).

The measurement of radon in the breath has been used extensively to estimate the amount of radium in the bodies of workers in the radium industries. Such measurements have in recent years been made by counting the alpha particles emitted per unit time by the radon and its decay products (1, 5). To measure the very low radon content of the breath of individuals who have not accumulated abnormal amounts of radium in their bodies, the alpha counting equipment must be very sensitive and stable. Apparatus which satisfies these requirements has been designed and built by the Atomic Energy Project of the National Research Council of Canada in Chalk River, Ont., and has made possible the present investigation.

The sampling method was developed with the following consideration in mind: "Fresh" atmospheric air contains variable concentrations of radon. As long as the radium content of the body is at its low normal level it will contribute to the breath only negligible amounts of radon which are not measurable. The radon content of the breath will be close to and governed by the radon content of the atmospheric air and will fluctuate with it. When the radium content of the body is increased it will contribute to the breath an increased amount of radon which will be constant and independent of fluctuations of the radon content of the air. It may still be within the range of the ordinary radon content of the air and therefore be veiled by this. It appears therefore necessary to control the factor of radon in the inhaled air.

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This we have done by having the subjects breathe radon-free air. In this way it is possible to investigate through analyses of radon in the breath the content of radium in the body in the range between normal and the amounts now regarded as the limits of tolerance.

Materials and Methods

The subjects were normal men between 18 and 54 years of age who had not at any time worked with radium or lived in the vicinity of a radium industry. Breath samples were taken between 3 and 5 p.m. on ordinary working days, three to five hours after the last meal, with the subjects on their ordinary diets.

Sampling

Because atmospheric air contains appreciable and variable quantities of radon the subjects were required to breathe radon-free air before and during sampling. In preliminary experiments it was found that the radon content of the expired air after periods of 5 min. and 30 min. of breathing radon-free air, was not measurably different. Accordingly, a standard time of 10 min. of breathing of the radon-free air was adopted.

The breath samples were collected in Pyrex aspirator bottles (6) of 2000 or 4000 ml. capacity fitted with pure gum-rubber stoppers and adapters which were coated with a solution of polystyrene. The bottles were cleaned with a 0.2% solution of Tween 80, then with 7% hydrochloric acid, and finally with 10% citric acid and distilled water, and were tested to make sure that no measurable radon generation and adsorption took place within the limits of our experimental conditions.

The radon-free air was obtained by allowing compressed air in a commercial cylinder to age for 28 days. The cylinder was fitted with a pressure reducing valve which in turn was connected to a demand valve. The outlet of the demand valve was connected to one arm of a T tube. The second arm of the T tube carried a B.M.R. mouthpiece, through which the subject breathed. The third arm led through a saliva-trap to the sample-collecting bottle. The outlet of the sample bottle was fitted with a nonreturn valve dipping into a beaker of water. The diameters of all connections were chosen so that resistance to breathing at rest caused no discomfort. The subject was comfortably seated with his nose clamped tightly for the duration of the experiment, which was 10 min.

High Vacuum System

Fig. 1 shows the high vacuum line for the transfer of the breath sample into the 1200 cc. ionization chamber.

The central electrode was insulated with Teflon and was shielded from the high voltage insulator with a grounded guard ring. The moisture trap was cooled with a mixture of dry ice and acetone. Before filling the chamber the system was thoroughly dried and degassed at a pressure between 12 and 14 μ of mercury.

The sample bottle was connected with the vacuum line through a tube filled with sodium hydroxide for the removal of carbon dioxide. Then, by operating the stopcocks, the breath sample was made to stream to the ionization chamber

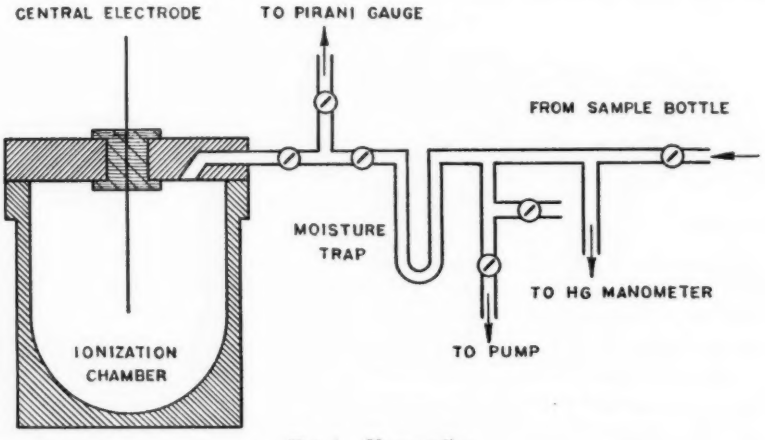

FIG. 1. Vacuum line.

at a rate of 60 cc. per minute. Simultaneously warm radon-free water was allowed to flow into the aspirator bottle, to displace the gas until atmospheric pressure was reached in the ionization chamber. The room temperature and the barometric pressure were recorded at the end of each operation.

Counting Equipment

The positive 1500 v. high tension supply was connected to the wall of the ionization chamber, while the central electrode was coupled to a head amplifier* via load resistance of 10^9 ohms. The first amplifying tube is a Sylvania Type 5638 pentode connected with a high anode load of 2.5 *M* and an anode current of 100 μa. The output of the head amplifier was then fed into a slow linear amplifier**. The slow linear amplifier simultaneously operated a numerator† with a recovery time of 10^{-1} sec. and a scaler with a recovery time of 5×10^{-6} sec. operating a register. Both the output of the numerator and the scaler were fed into separate pens of an Esterline Angus recorder. The comparison of the counts obtained from the two registers and the frequency distribution shown on the chart of the Esterline Angus recorder were used to assess the reliability.

The frequency response of the main amplifier can be chosen at will by varying independently time constants which control the lower and upper

* *Type 1408 designed by the Electronics Branch, National Research Council, Atomic Energy Project, Chalk River.*

** *Type 1433 designed by the Electronics Branch, National Research Council, Atomic Energy Project, Chalk River.*

† *Numerator Mark II designed by the Electronics Branch, National Research Council, Atomic Energy Project, Chalk River.*

frequency limits and, to achieve a maximum ratio of signal to noise in our case, these time constants were chosen to have values of 1 msec. and 10 msec. respectively.

A voltage discriminator is provided at the output of this equipment to select the minimum pulse amplitude to be registered, and this was set to accept pulses greater than 30 v. The noise level at the discriminator was 10 v.

It was not established whether the background counts were spurious pulses originating in the electronic equipment, or were due to contamination of the ionization chamber. This point will be the subject of further investigation.

In this study two complete sets of equipment were used which differed somewhat in background and amplification. The background was determined for each set at frequent intervals between analysis, with the chamber filled with radon-free air. The counting efficiency of each set was determined by filling the chamber with radon extracted from a standard solution (supplied by the U.S. National Bureau of Standards) containing 10^{-11} c. of radium, or from a solution containing 5×10^{-13} c. obtained by dilution of the former. The backgrounds were counted over periods of from 15 to 48 hr. For equipment *A* the background averaged 7.35 counts per hour, with standard deviation of 1.6 for 12 observations; the net counts per hour for the standard of 10^{-11} c. averaged 1170 ± 27 (S.D.). For equipment *B* the background averaged 2.8 counts per hour ± 1.0 (S.D.), and the net counts per hour for the 10^{-11} c. standard were 842 ± 41 (S.D.). Calibration figures with the diluted radium standard were approximately proportional. Unknown samples were counted for 9 to 17 hr.

The total counts per hour were corrected for background as determined shortly before and after the experiment. The breath radon at the time of delivery was then determined from the previous calibration after correcting for decay, and expressed in curies per liter of expired air at 37° C. saturated with water vapor, and at the prevailing barometric pressure (B.T.P.S.) (4). A correction was also made for the "external dead space" in the breath sampling apparatus.

Results

The radon content of expired air as found in 17 experiments on 15 normal subjects is shown in Table I. The quantities found were so small as to be

TABLE I

RADON CONTENT OF EXHALED AIR OF NORMAL SUBJECTS

Equipment	No. of expts.	No. of subjects	Average Rn content ± S.E. per liter as curies $\times 10^{-14}$
A	13	12	1.54 ± 0.22
B	4	4	1.36 ± 0.81
	Combined average		1.5 ± 0.24
			$P < 0.001$

hardly significant in any one experiment, but the average of all experiments shows a quantity significantly greater than background.

Discussion

Experimental Errors

Tests were made of the sampling procedure with the subject breathing oxygen from a cylinder for 10 min. (instead of compressed air). The N_2 content of the sample bottles was found to be less than 1%. This means that the amount of air which had not been flushed out by the breath and the amount of air which might have leaked into the sampling circuit must have been considerably less than 1% of the sample. Depending on the radon content of our room air, it would account for zero to 2.3×10^{-15} c. of radon per liter of breath.

Contamination with air from the paranasal sinuses, or from the stomach, might also add radon, since such might be expected to contain at least as much radon as room air. Contamination from these sources in a single experiment cannot be ruled out but must be very small in the series, and cannot be a systematic error, as shown by the tests just described.

Natural Sources of Radon

All rocks, and therefore soil and water, have been found to contain varying but measurable amounts of radium. As the radium decays the surface layers of the earth are continuously impregnated with radon and give off radon into the atmosphere and the water.

Depending on weather conditions and composition of the soil, the radon content of the atmospheric air varies over wide ranges. The literature reports from 0.7 to 4×10^{-13} c. per liter (8), and in our well ventilated laboratory we have found various values from less than 10^{-14} to 2.3×10^{-13} c. of radon per liter of air. We estimate that this latter amount would contribute to radon in expired air 1 to 2×10^{-14} c. per liter of air. This estimate is based on the distribution coefficient of radon between body fluids and air (0.17) (7), and on the decrease of the elimination rate of radon after 10 min. of aeration of the lungs with radon-free air (9).

The contribution of the other factors can be estimated as follows: 1.5×10^{-10} gm. of radium in the body (5) would contribute a maximum of 2×10^{-15} c. of radon per liter of breath. Additional transient radium in food, calculated on 25 gm. of mineral intake per day, would contribute 0.3×10^{-15} c. of radon per liter of breath. As there are no experimental data available on the radon content of food this estimate is based on the average radium content in rocks, which may be assumed to be 10^{-12} gm. of radium per gm. (3).

Radon dissolved in food may be estimated from the above radium content at equilibrium and would contribute 1.7×10^{-15} c. per liter of breath. Lake Ontario drinking water in equilibrium with 2.3×10^{-13} c. of radon per liter of air would contribute only a negligible amount of radon to breath (2). Hence the radium level in the body plus the radium ingested with food and water would contribute about 4×10^{-15} c. per liter of breath.

Under our conditions it appears that the radon in atmospheric air establishes a concentration of radon in the body that supplies a larger proportion of the exhaled radon than radon from all other sources put together. However, great fluctuation of the proportions may be expected, depending on local and atmospheric conditions.

The above estimates are oversimplified approximations, useful to give an idea of the order of magnitude which we can expect from radon in breath under our local conditions. This order of magnitude is in good agreement with our experimental findings.

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THE TYPE SPECIFIC CAPSULAR ANTIGEN OF *PASTEURELLA MULTOCIDA*[1]

By G. R. Carter

Abstract

It has been demonstrated by means of two precipitation techniques and a capsular swelling test that *Pasteurella multocida* possesses a type specific capsular antigen presumably polysaccharide in nature. The antigens employed in the precipitation tests were recovered from virulent cultures by two techniques. In the first the capsular antigen was released by organisms suspended in normal saline and in the second the specific antigen was recovered by an extraction method. Antisera were produced in rabbits and chickens by the inoculation of formolized culture suspensions. On the basis of the type specific capsular antigen, three distinct serological types of *P. multocida* have been identified.

Introduction

Active and passive immunizing agents have been employed for the control of pasteurellosis for a number of years. Although it is known that there are several serological types of *Pasteurella multocida*, because of the difficulty of identifying types, in most laboratories, strains for the preparation of bacterins and antisera are selected on zoological rather than serological grounds. The primary purpose of this investigation, aside from elucidating the antigenic nature of *P. multocida*, was to develop a satisfactory technique for the routine typing of members of the hemorrhagic septicaemia group.

The serological methods which have been applied to *P. multocida* in the past have usually depended upon immunological reactions involving both specific and nonspecific factors and consequently strong cross reactions have made difficult the unequivocal identification of types. That a type specific antigen is associated with the capsule was suggested by the work of Hoffenreich (5) in 1928.

Literature Review

Hoffenreich extracted a polysaccharide substance from the capsules of *P. multocida*, which resembled in its physical, chemical, and serological properties, the soluble specific capsular polysaccharides isolated by earlier workers from the capsules of a number of pathogens. Employing the method of Boivin and Mesrobeanu, Pirosky (9) extracted polysaccharide antigens from smooth forms of *P. multocida* and by means of precipitation tests he demonstrated their serological specificity and suggested that their specificity might make possible a serological classification of the group.

The four principal colonial variants of *P. multocida* have been described by Hughes (6) and Webster and Burn (17) as mucoid, fluorescent, intermediate, and blue. Although other variants have been reported (4), Hughes, and Webster and Burn's descriptions of the dissociation pattern were found sufficiently comprehensive for this study.

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Contribution from the Division of Animal Pathology, Science Service, Dominion Department of Agriculture, Animal Diseases Research Institute, Hull, Que.

A number of investigators have employed a variety of methods in an attempt to accomplish a serological classification of the group. Their results have been comprehensively reviewed by Rosenbusch (14). Some idea of the difficulty that has been encountered is evidenced by the fact that Cornelius (2) was only able to classify 17 of his 26 strains, and Yusef (18) could identify only 14 of his 21 cultures.

Rosenbusch and Merchant (15) divided their strains into three principal groups on the basis of agglutination, and action on xylose, arabinose, and dulcitol. Seven of their 39 strains were classified as variants. Little and Lyon (7) found that their 30 strains fell into three distinct groups when typed by means of slide agglutination and serum protection tests. By means of serum protection tests, Roberts (12) found that his 37 strains comprised at least four groups.

Materials and Methods

Origin and Maintenance of Cultures

All cultures were maintained on airtight beef-infusion agar slants stored at room temperature. When it was necessary to maintain cultures for long periods transfers were made at monthly intervals. Only strains conforming with the description of *P. multocida* as given in Bergey's manual (1), except for the production of hydrogen sulphide, were included in this study. The animal origin and proposed serological designation of the four cultures employed to demonstrate the type specific capsular antigen are given in Table I.

TABLE I

PROPOSED SEROLOGICAL DESIGNATION AND ANIMAL SOURCE OF *P. multocida* STRAINS

Culture No.	Serological designation	Animal origin
1000	*A*	Buffalo
1001	*A*	Bovine
1002	*B*	Buffalo
1003	*C*	Canine

Preparation of Antisera

Only cultures yielding a large proportion of mucoid, fluorescent, and intermediate colonial forms produced sufficient amounts of specific capsular antigen for the preparation of the specific sera referred to in Table II. Avirulent cultures yielding a large proportion of blue variants on clear tryptose horse serum agar were passed in either mice or chick embryos. After one or more passages they were observed to produce a large proportion of fluorescent

and intermediate forms. The immunizing cultures were grown on tryptose horse serum (10%) agar containing 0.2% dextrose and 1.0% sucrose. After incubation at 37° C. for 24 hr., the growth from each plate was washed twice in normal saline and then suspended in sufficient 0.25% formalized buffered saline (pH 7.0) to produce a turbidity corresponding to tube No. 4 of McFarland's nephelometer. Before immunization was begun all rabbits and chickens were tested for the presence of precipitins against *P. multocida*.

Satisfactory titers were obtained in some rabbits after 12 injections. Rabbit antisera were employed for the precipitation tests referred to in Table II. Sera from several chickens were used for the capsular swelling tests after the administration of six to eight inoculations of antigen (16). With rabbits the immunizing dose ranged from 1.0 ml. to 5.0 ml. and for chickens a uniform dose of 3.0 ml. was administered. Several additional injections of live organisms administered in decreasing dilutions were required to produce antisera sufficiently potent for MacPherson's precipitation test. The intervals between injections varied from three to seven days.

Precipitation Tests

(1). This method was adapted from Pittman's (10) technique of demonstrating the type specific capsular antigen of *Hemophilus influenzae*. The antigen for this test consists of what is presumed to be soluble polysaccharide material, released by capsulated organisms when suspended in normal saline. Because the capsular material of mucoid strains appeared to be less soluble in saline than that of fluorescent strains, only nonmucoid strains were typed by this test. Each culture listed in Table I was inoculated heavily on a tryptose horse serum agar plate. After incubation at 37° C. for 24 hr., the growth was washed off with 3.0 ml. of buffered saline and the suspension heated at 56° C. for one hour. The organisms were centrifuged out and by the method of doubling dilutions the supernatants were diluted to a final dilution of 1 : 64 in buffered saline. The antisera in 0.2 ml. amounts were added to 0.2 ml. amounts of the supernatant dilutions and the mixtures incubated at 37° C. for one hour and then left at 4° C. for 18 hr. prior to reading. A positive test consisted of a precipitate which could be readily discerned when the tubes were shaken sharply. Control tubes contained 0.2 ml. of the buffered saline diluent and 0.2 ml. of the specific antiserum. Data in regard to the dilutions and identity of antisera and antigens are given in Table II.

(2). The technique evolved by MacPherson (8) for typing *H. influenzae* separated the strains listed in Table I into the same categories as the aforementioned method and had the advantage of involving the use of considerably less antiserum. All strains producing fluorescent, intermediate, and blue colonies on agar were typable by this method. By means of this method MacPherson was able to type nontypable (by the capsular swelling technique), nonencapsulated, derivative strains of *H. influenzae*. Except that the cultures of *P. multocida* were cultivated on tryptose horse serum agar rather than on Fildes or Levinthal agar, the technique employed was identical to that

TABLE II

RESULTS OF PRECIPITATION TESTS DEMONSTRATING THE SPECIFICITY OF THE CAPSULAR ANTIGEN OF *P. multocida* STRAINS

Serum	Antigen	Final dilution of supernatant fluid						Controls
		1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	
1000 Type *A*	1000	++	++	++	++	++	+	−
	1001	++	++	++	++	+	±	
	1002	±	−	−	−	−	−	
	1003	±	−	−	−	−	−	
1002 Type *B*	1000	±	−	−	−	−	−	−
	1001	±	−	−	−	−	−	
	1002	++	++	++	++	++	+	
	1003	±	−	−	−	−	−	
1003 Type *C*	1000	±	−	−	−	−	−	−
	1001	±	−	−	−	−	−	
	1002	±	−	−	−	−	−	
	1003	++	++	++	++	++	++	

NOTE: ++ = *Precipitate.*
± = *Slight precipitate.*

described by MacPherson. As in the previously described test the antigen consists of the type specific soluble capsular substance.

Capsular Swelling Tests

The capsulated organisms for this test were obtained from 8-15 hr. tryptose broth cultures and from 24 hr. 10-day chick embryo cultures. The hanging drop mounts were prepared as follows: 1. A ridge of petrolatum in the outline of the perimeter of a cover slip was applied to a glass microscope slide with a hypodermic syringe. 2. Two loopfuls of a given antiserum were mixed with a loopful of Loeffler's methylene blue solution on a glass slide. 3. The bacterial suspension was prepared by the addition of one or two drops of broth, or yolk from the chick embryo culture to 5.0 ml. of normal saline. A small loopful of this suspension was added to the antiserum–methylene blue mixture. After thorough mixing, one loopful was transferred to a cover slip which was inverted over the perimeter of petrolatum thus giving a hanging drop. The examinations for capsular swelling were greatly facilitated by the use of double-size cover slips from which were suspended several drops each containing a specific antiserum. 4. Microscopic examinations of the moist preparations were made after the mixtures had stood at room temperature for periods of 15 and 30 min. Antisera were diluted as was indicated.

As swelling takes place the capsule becomes more prominent and opaque, and its outer edge becomes more clearly outlined.

Results

The results of the precipitation tests in which the capsular antigen was washed from the organisms are shown in Table II and they demonstrate very

clearly the type specificity of the capsular antigen. The type identity of the strains listed in Table I was also demonstrated by capsular swelling tests and by MacPherson's precipitation test.

Discussion

By means of the typing methods described in this study it should be possible in the near future to determine the incidence of the different serological types in the various domesticated species. Besides obtaining this information, data relative to the dissociation status of fresh isolates and the presence or absence of disease should be recorded. Webster and Burn (17) have observed that mucoid strains are usually associated with the carrier state in rabbits, and the author has noted that such is often the case in swine. Transformation of the mucoid form to the virulent fluorescent phase does not appear to have been accomplished. In view of these considerations it would seem that the mere isolation of *P. multocida* would not be sufficient grounds for inferring its potential pathogenicity. Information derived from a comprehensive survey should afford a rational basis for the selection of strains for the preparation of bacterins and antisera.

In some instances the use of type specific antisera for prophylactic and therapeutic purposes might be of value. The use of polyvalent Quellung antisera for the rapid diagnosis of Pasteurellosis should be explored.

Information derived from this investigation and from the studies of other workers throw considerable light on the antigenic nature of *P. multocida*. There appear to be two principal antigenic components:

1. A type specific, soluble polysaccharide antigen consisting of or associated with the capsule; and

2. A somatic antigen common to all members of the species. That the capsular antigen is polysaccharide in nature may be adduced from the following evidence: (1) The identification by Hoffenreich of a polysaccharide substance associated with the capsule. (2) The tendency of capsules to swell in the presence of homologous antiserum. (3) MacPherson's technique for typing *Hemophilus influenzae* by the extraction of specific polysaccharide has been employed with *P. multocida* and has yielded results identical with those obtained with the first technique described in this study. (4) The capsular substance has been observed to be antagonistic to phagocytes (3).

Considering that it is the capsular antigen which elicits the greatest immune response in mice (11), one would expect a close correlation between type and immunological specificity. That such is the case has been demonstrated by vaccination and challenge experiments in mice and will be published as a separate report.

The difficulties which have been encountered in the past in the typing of *P. multocida* appear to have been due for the most part to the complex dissociation pattern of the organism. *P. multocida* may yield in the same culture varying numbers of fluorescent, intermediate, and blue variants. Cultures containing a large proportion of mucoid or fluorescent forms are almost

inagglutinable although because they possess large amounts of capsular antigen, they are highly satisfactory for Quellung tests. Strains producing flocculent growth in broth, a large proportion of blue variants on agar, and manifesting low virulence for mice and chick embryos, often undergo spontaneous agglutination and yield a very small amount of specific capsular antigen. Antigens prepared from these cultures were found to be unsatisfactory for both agglutination and precipitation tests. Because mucoid strains as compared with fluorescent strains are generally less virulent, they are difficult to type by means of serum protection tests in mice.

Because Roberts, Rosenbusch and Merchant, and Little and Lyon have employed different numbers to designate what appear to be identical types (13), and because it is felt that the methods of typing described in this study are less subject to error than previous techniques described, a new alphabetical designation is proposed.

That more than three serological types of *P. multocida* exist is suggested by the work of Cornelius, Yusef, and Roberts. An examination of more than 40 cultures from a variety of animal species indicate that Types *A*, *B*, and *C* occur most frequently. In a report to follow it is planned to establish the type identity and animal source of a large number of strains recovered in Canada and the United States.

Summary

Three distinct serological types of *P. multocida* have been identified by means of precipitation tests in which the antigen consisted of a soluble capsular substance presumably polysaccharide in nature. The type specificity of this capsular antigen has been confirmed by a capsular swelling technique and this method is recommended for the routine typing of *P. multocida*.

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STUDIES ON PASSIVE IMMUNITY IN POLIOMYELITIS

III. PRODUCTION OF IMMUNE SERUM TO LANSING POLIOMYELITIS VIRUS IN RHESUS MONKEYS[1]

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WITH HISTOLOGICAL OBSERVATIONS

By W. L. Donohue[3] and R. C. Ritchie[3]

Abstract

Thirty rhesus monkeys have been repeatedly injected with Lansing poliomyelitis virus by the intramuscular route. Antibody, as measured by a virus-neutralization test in mice, rapidly developed in the serum. About 3 liters of immune serum with a 50% neutralizing titer of $10^{-3.0}$ or higher were collected with a view to fractionation. Antibody levels tended to be maintained during rest periods when no injections were given. The evidence suggests that, on the whole, antibody levels were higher in animals receiving virus incorporated in paraffin oil adjuvant than in those receiving virus suspended in water. Certain individual monkeys receiving virus in water responded as well, however, as monkeys receiving virus in adjuvant.

Introduction

It has been shown by Morgan and her colleagues that rhesus monkeys given repeated intramuscular injections of poliomyelitis virus develop serum antibody and eventually become resistant to a cerebral challenge of homologous virus (4, 8, 10, 11, 12, 13, 14). The most detailed studies have been carried out with the Lansing strain, for antibody levels can be readily and fairly accurately measured by the inoculation of mice with a fixed amount of virus and serial dilutions of serum (2, 3, 7). It has been shown that the degree of antibody response is related to the amount of virus inoculated, and the maximal response was found in animals inoculated on four occasions with a 20% suspension of infected monkey cord (12). Serum antibody levels, expressed in the form of the 50% neutralizing titers of the serum, were as high as 1 : 8000 in some of these animals. An increased dosage of virus did not lead to the development of significantly higher antibody levels. The important observation was made that vaccinated animals in which the antibody titers reached 1 : 1000 or higher were resistant to cerebral challenge, whereas those with lower serum antibody levels were not.

Antibody levels tend to fall progressively during rest periods in vaccinated animals, but rise promptly after a "booster" injection (1, 10, 13).

Attempts have been made to raise antibody levels by the incorporation of the inoculum with an adjuvant substance. Thus, Morgan, Howe, and

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Bodian (14) inoculated monkeys with virus incorporated in "Falba" and paraffin oil. If mycobacteria were added, many of the animals died of "allergic encephalomyelitis" (9). If no mycobacteria were added to the inoculum, the animals survived, but they did not develop high antibody levels.

Encouraged by results in experiments with rabies virus (5, 6), Freund and Ward have also investigated this question. It has been reported by Ward *et al.* (18, 19) that the incorporation of Lansing virus in paraffin oil (Bayol) has an enhancing effect on antigenicity as shown by antibody development. For example, in samples removed one month after injection, mean neutralizing titers were about 10 times higher in the sera of monkeys inoculated with virus in adjuvant than in a group receiving virus in saline. As further injections of virus (in saline) were given, differences in titer became less evident. Ward *et al* found that some monkeys developed titers of over 1 : 10,000; in one animal that had developed nonparalytic poliomyelitis before immunization, the exceptionally high titer of 1 : 41,000 was found.

Salk *et al.* (17) have confirmed the rapid production of antibody after as few as two injections of virus incorporated in adjuvant.

In these laboratories, we are studying various aspects of the problem of passive immunity in poliomyelitis (15, 16). Of particular interest is the question of fractionation of animal immune serum to determine whether the virus neutralizing effect is associated with the gamma or other protein fraction. For such an investigation, considerable quantities of immune serum of high antibody content were required. In view of the above quoted experience, it appeared that substantial amounts of hyperimmune serum could be obtained from rhesus monkeys subjected to repeated intramuscular injections of Lansing virus.

This paper describes experiments in which 30 monkeys have been so injected. Some of the animals were vaccinated with virus suspended in water, others with virus in adjuvant. Some animals were survivors from titration experiments and had received subinfective amounts of Lansing virus by the cerebral route; other animals had not been exposed to Lansing virus prior to immunization. Many monkeys died before substantial amounts of immune serum were obtained, nevertheless, over 3 liters of serum with a 50% neutralizing titer of 1 : 1000 or higher were collected. This serum has now been fractionated by Dr. J. W. Palmer and Mr. Leo Zuckerman of E. R. Squibb & Sons, New Brunswick, N.J. The results of this work are being reported separately.

Technical Methods

Technical methods were, in general, similar to those used by the workers referred to above and, as we have previously given details, only certain additional points need be covered here (16).

The monkeys were vaccinated with four pools of Lansing virus (Nos. 2, 5, 7, and 8) prepared from the cervical and lumbar enlargements of the spinal cords of monkeys developing paralysis after thalamic inoculation (Table I). The Lansing virus was originally supplied by Dr. Howard Howe in the form

TABLE I

POOLS OF LANSING VIRUS USED FOR IMMUNIZATION OF MONKEYS

Pool	Inoculum in monkeys yielding cords for pool	Numbers of monkeys yielding cords for pool	Pool prepared as watery suspension of following strength	PD_{50} titer of pool determined in monkeys
2	1 : 100 dilution of monkey cord (Dr. Howe's Lansing pool VIII)	26	25%	$10^{-4.15}$
5	1 : 100 dilution of cord (our pool No. 2)	20	20%	Between $10^{-4.5}$ and $10^{-5.0}$
7	1 : 100 dilution of cord (pool No. 5)	29	25%	Over $10^{-4.0}$
8	1 : 100 dilution of cord (pool No. 7)	32	25%	$10^{-5.4}$

of monkey cord representing his pool VIII. Our pools Nos. 2, 5, and 7 were titrated by the thalamic inoculation of serial 10-fold dilutions of spinal cord, usually from $10^{-3.0}$ to $10^{-5.0}$ in groups of four monkeys. With such small numbers of animals, only an approximate PD_{50}* could be calculated, but this was sufficient for our purposes. With all three pools, paralysis occurred in monkeys inoculated with cord diluted $10^{-4.0}$, and the virus contents were therefore judged to be satisfactory. In the titration of pool No. 8, 10 monkeys were injected in each group and the titer could thus be more accurately determined; it was found to be $10^{-5.4}$. The details of these four pools are shown in Table I.

Inoculum for the vaccination programs was prepared by suspending pool material in distilled water or in paraffin oil adjuvant. In most of the experiments described in this paper, monkeys were vaccinated with a constant volume of 2 ml. of 12.5% suspension of cord in distilled water or in adjuvant, so that 0.25 gm. of nervous tissue was inoculated on each occasion. For the preparation of adjuvant, Bayol F and Arlacel A were mixed in the proportion of nine parts of the former to one part of the latter. Equal parts of pool and adjuvant mixture were then mixed in a Waring blendor, until emulsification reached the required stage. This procedure was personally demonstrated to one of us in Dr. Freund's laboratory.

All injections were given intramuscularly in the calf or pectoral muscles, and not more than 1 ml. was injected in any one site. Most of the injections were given at intervals of two weeks.

Bleedings were at first carried out by heart puncture, but later in the program blood was taken from the femoral vessels. Generally about 40 ml. were

** One PD_{50} is the dose paralyzing 50% of test monkeys inoculated in the thalami with 0.8 ml. of dilutions of infected monkey cord.*

withdrawn on each occasion. Larger quantities can be removed, but this may lead to death from blood loss.

Tests in mice for virus neutralizing antibody were carried out as follows: The sera were diluted in saline in serial 10-fold steps and virus pool was added, usually to a final dilution of $10^{-2.7}$. Two mouse pools were used in this work, pool No. 1 having a cumulative LD_{50}* titer of $10^{-4.03}$, and pool No. 2 of $10^{-3.93}$ (16). Accordingly, in tests in which pool No. 1 was used, each mouse inoculated with serum–virus mixture received on the average 21 LD_{50} of virus, and in tests with pool No. 2 the amount was 17 LD_{50}. A few tests, specially indicated in the tables, were carried out with pool No. 2 at a final dilution of $10^{-2.0}$ (85 LD_{50}). Serum–virus mixtures were injected cerebrally in groups of 8 or 10 mice.

Antibody titers of sera are expressed in terms of the 50% neutralizing titer, which is that dilution of serum, before admixture with an equal volume of virus, that protects 50% of mice from death. For example, if 50% of mice are protected when inoculated with virus and serum diluted $10^{-4.0}$, the 50% neutralizing titer of the serum is taken as $10^{-4.0}$ even though the final dilution of serum is actually $10^{-4.3}$. As is customary in this work, titers will be expressed in the form of logarithms of reciprocals of dilutions giving 50% protection, e.g., a titer of $10^{-4.0}$ is expressed as 4.0.

Throughout this work, 50% end points of virus or serum have been calculated by plotting probits of mortalities against dilutions of virus or serum, drawing a straight line between the points, and reading off the dilution where 50% of animals would be expected to die. Unpublished studies indicate that the estimates of LD_{50} obtained by this method are in close agreement with those obtained by the Kärber or Reed and Muench method. These studies also show that when titrations of a single immune serum are performed on repeated occasions, 95% of the estimated 50% neutralizing titers will fall within a range of 0.5 log units. This degree of accuracy was adequate for the present purposes, but in a more detailed study it would be necessary to use larger numbers of animals.

Results

Some 30 monkeys were vaccinated in four separate immunization programs. In the first three groups (*A*, *B*, and *C*) the monkeys had all survived, before the commencement of vaccination, the cerebral inoculation of Lansing virus pools in titration experiments without developing paralysis. Nonparalytic infection with Lansing virus is known to be rare in the monkey and none of these animals was noted to have any such illness. The possibility remains that some of these animals did however suffer from a "subclinical" attack. The animals in Group D had not been exposed experimentally to Lansing virus before the vaccination program was begun.

Of the 30 monkeys, 12 were vaccinated with virus suspended in water on all occasions, and the remainder received virus in adjuvant on the first occasion

** One LD_{50} is the dose killing 50% of mice inoculated cerebrally with 0.03 ml. of dilutions of virus pool.*

and usually subsequently. The monkeys in Group A received a large dose of Lansing virus (1 gm.) at the beginning of the immunization program, and thereafter received injections every two weeks except for an interval in the summer. The animals in the other groups did not receive a large dose of virus initially, but were vaccinated throughout by means of injections given at intervals of two weeks.

Group A

This group consisted of nine monkeys that survived without apparent ill effect the cerebral inoculation, in January 1950, of various dilutions of monkey pool No. 1 (Table II). This pool was not used in any of the vaccination programs discussed in this paper as it was of low virus content. In March of the same year the animals were injected intramuscularly with 1.0 gm. of monkey cord (pool No. 2). Four animals received virus suspended in water and the remainder, virus in adjuvant. Bleedings were taken in Weeks 3, 5, and 10.* The antibody level in Weeks 3 and 5 was only above 3.0 in 1 monkey. These particular tests were not planned to measure smaller amounts of antibody. However, it is of interest that 10 weeks after vaccination, all the five monkeys vaccinated with virus in adjuvant had antibody titers of 3.0 or higher, whereas the titers in the four animals receiving virus in water were all below 3.0. In Week 10 a second course of vaccination was commenced, and three "boosters" were injected at approximately two-weekly intervals. The antibody levels in Weeks 13 to 16 were low in two of the monkeys receiving virus in water, but in all the remaining monkeys the titers were above 3.0 and often above 4.0. Two monkeys died shortly after the completion of this course. Blood was taken in Week 23, nine weeks after the last injection of virus; the antibody levels in these samples appeared to have dropped in the monkeys vaccinated with virus in water, but were relatively unaffected in those receiving virus in adjuvant. In Week 23 the remaining seven monkeys were started on a further course of injections. Three of the animals died after from one to three such injections, but four survived and received either 2.25 or 2.5 gm. of cord in Weeks 23 to 41. The antibody titers in these four animals from Week 26 onwards were all above 3.0, and mostly over 4.0. Some titers were 5.0 or higher. These four monkeys showed consistently high antibody levels during the third course of vaccination. Over one liter of immune serum with a titer of 3.0 or higher was obtained from the animals in this group.

Group B

This group consisted of five monkeys. Three of these had survived, without apparently becoming sick, the inoculation of dilutions of pool No. 1, and the other two had received dilutions of pool No. 2. All monkeys were then vaccinated by means of injections at intervals of two weeks (Table III). Three such injections were given in the first course; one animal died at this stage. The other four animals all promptly developed antibody titers of 3.0

** Adopting the convention used by Morgan, the week in which vaccination was started is described as week 0 and subsequent weeks as 1, 2, 3, and so forth.*

or higher. After a nine week rest, the four surviving monkeys were bled; antibody titers after the rest (Week 13) were the same as those in the previous sample taken seven weeks before (Week 6) or somewhat higher. Starting in Week 13, injections were again given approximately every two weeks. All bleedings showed antibody titers substantially above 3.0. The only animal injected with virus suspended in water developed particularly satisfactory antibody levels. Titers of over 5.0 were obtained on two occasions. The antibody response in this monkey (No. 810) may be compared with that of

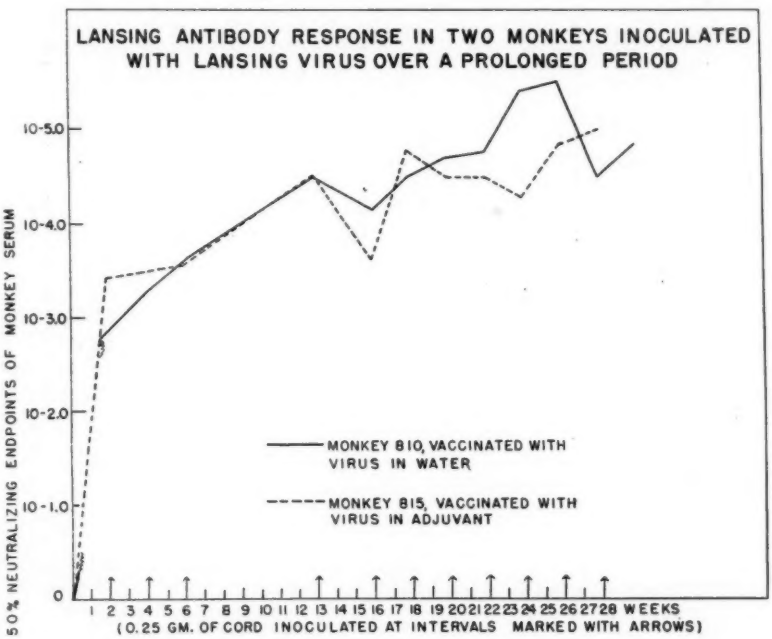
LANSING ANTIBODY RESPONSE IN TWO MONKEYS INOCULATED WITH LANSING VIRUS OVER A PROLONGED PERIOD
50% NEUTRALIZING ENDPOINTS OF MONKEY SERUM
10-5.0
10-4.0
10-3.0
10-2.0
10-1.0
0
MONKEY 810, VACCINATED WITH VIRUS IN WATER
MONKEY 815, VACCINATED WITH VIRUS IN ADJUVANT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 WEEKS
(0.25 GM. OF CORD INOCULATED AT INTERVALS MARKED WITH ARROWS)

FIG. 1. Lansing antibody response in two monkeys inoculated with Lansing virus over a prolonged period.

No. 815 inoculated with virus in adjuvant, by reference to Fig. 1. It will be seen that the character and degree of the responses are remarkably similar.

Group C

Five monkeys constituted this group. These had survived without illness the thalamic inoculation of dilutions of pool No. 7 or pool No. 5 in titration experiments (Table IV). Three of the animals died during the vaccination course which consisted of injections every two weeks. Antibody developed rapidly in the serum of these monkeys, even after a single injection. No difference in antibody titers could be detected in this group in monkeys receiving virus in water as compared to those receiving virus in adjuvant.

TABLE

GROUP A MONKEYS: RESPONSE TO REPEATED

Monkey No.	Survived cerebral inoculation of following dilution of infected cord (Pool No.1) Jan. 1950	Vaccine suspended in	First course of vaccination				Second course of vaccination (two-weekly "boosters")				
			Week 0 Mar. 1950	Week			Weeks 10, 13, 14	Week			
				3	5	10		13	14	16	23
			Amount of cord,* gm.	Serum titers†			Amount of cord,** gm.	Serum titers†			
808	$10^{-3\cdot0}$	Water	1.0	<3.0	<3.0	2.21	0.75	3.84	3.75	4.08	3.15
809	$10^{-3\cdot0}$	Water	1.0	<3.0	<3.0	2.05	0.75	3.5 (85)	<3.0 (85)	<3.0 (85)	2.0
814	$10^{-4\cdot0}$	Water	1.0	<3.0	<3.0	1.38	0.75	<3.0 (85)	<3.0 (85)		
816	$10^{-4\cdot0}$	Water	1.0	<3.0	<3.0	1.5	0.75	3.0 (85)	3.6 (85)	3.2 (85)	2.5
838	$10^{-2\cdot0}$	Adjuvant	1.0	<3.0		4.0 (85)	0.75	3.78	5.0	5.0	5.05
811	$10^{-3\cdot0}$	Adjuvant	1.0	<3.0	<3.0	4.775	0.75	4.5	5.0	4.8	4.275
812	$10^{-3\cdot0}$	Adjuvant	1.0	3.0	4.5	3.36	0.75	4.25	4.0	4.78	5.0
817	$10^{-4\cdot0}$	Adjuvant	1.0	<3.0		3.73	0.75	4.5	3.86	4.25	4.64
818	$10^{-4\cdot0}$	Adjuvant	1.0	<3.0	<3.0	4.0	0.75	4.38	4.48	4.48	5.79

* *Pool No. 2.*

** *Pool No. 5.*

† *Amount of virus used in neutralization tests, approximately 20 LD_{50}; except in a few instances specified where 85 LD_{50} were used.*

†† *Pool Nos. 7 and 8.*

TABLE

GROUP B MONKEYS: RESPONSE TO REPEATED

Monkey No.	Survived cerebral inoculation of following dilution of infected cord	Vaccine suspended in	First course of vaccination (two-weekly "boosters")					
			Weeks 0, 2, 4 May-June	Week				
				0	2	4	6	13
			Amount of cord,* gm.	Serum titers†				
810	$10^{-2\cdot0}$ (Pool No. 1, Jan. 1950)	Water	0.75	0	<3.0	3.23	3.63	4.5
815	$10^{-4\cdot0}$ (Pool No. 1, Jan. 1950)	Adjuvant	0.75	0	3.38	3.5	3.6	4.5
824	$10^{-5\cdot0}$ (Pool No. 1, Jan. 1950)	Adjuvant	0.75	0	0	>3.0		
A43	$10^{-4\cdot0}$ (Pool No. 2, Mar. 1950)	Adjuvant	0.75	0	3.0	4.15	4.23	4.5
A250	$10^{-5\cdot0}$ (Pool No. 2, Mar. 1950)	Adjuvant	0.75	0	>3.0	4.8	4.45	4.5

* *Pool No. 5.* † *See footnote Table II.* ** *Pool Nos. 7 and 8.*

II

INTRAMUSCULAR INJECTION OF LANSING VIRUS

Third course of vaccination (Two-weekly "boosters")									Outcome of experiment	Volume of serum with titer of 3.0 or higher collected, ml.
Weeks 23-41	Week									
	26	28	30	32	34	36	38	41		
Amount of cord,†† gm.	Serum titers†									
2.25	3.86	5.0	>5.0	4.09	3.53	5.0	4.35	3.375		262
2.25	3.0	3.65	3.0	3.65	3.22	3.22	3.6	3.9		206
									Died of TB Week 18	
0.75	<3.0	3.75							Died of TB Week 30	60
									Died of pneumonia Week 23	58
0.25									Died of emphysema Week 26	39
2.5	4.18	4.78	4.78	4.78		4.5	4.5	4.27	Died of TB Week 43	287
0.5	5.0								Bled out Week 26	91
2.25	4.48	4.48	4.48	4.48	4.6	4.62	4.5	4.5	Bled out Week 41	173

III

INTRAMUSCULAR INJECTIONS OF LANSING VIRUS

Second course of vaccination (two-weekly "boosters")									Outcome of experiment	Total volume of immune serum collected, ml.
Weeks 13-30	Week									
	16	18	20	22	24	26	28	30		
Amount of cord,** gm.	Serum titers†									
2.25	4.15	4.5	4.7	4.775	5.38	5.51	4.5	4.84		258
2.125	3.6	4.78	4.5	4.5	4.27	4.81	5.0		Died of TB Week 29	272
									Died after bleeding Week 4	
2.25	4.3	3.375	3.63	4.0	3.4	3.4	4.05	4.0	Died of TB Week 30	246
2.25	4.05	4.13	4.48	4.32	4.45	>5.0	3.75	5.0		208

TABLE IV

GROUP C MONKEYS: RESPONSE TO REPEATED INTRAMUSCULAR INJECTIONS OF LANSING VIRUS

Monkey No.	Survived cerebral inoculation of following dilution of infected cord	Vaccine suspended in	Course of vaccination (two-weekly "boosters")									Outcome of experiment	Total volume of immune serum collected, ml.
			Weeks 0-14	Week									
				0	2	4	6	8	10	11	13	15	
			Amount of cord,* gm.	Serum titers†									
A116	$10^{-5.0}$ (Pool No.7, Aug. 1950)	Water	1.75	0	1.67	2.25	2.77	2.07		3.98	5.0	4.5		39
A118	As for A116	Water	1.5	0	2.5	2.5	3.33	4.0		4.63			Died after bleeding Week 11	63
A119	As for A116	Water	0.75	0	0	3.1							Died Week 4	
A82	$10^{-5.0}$ (Pool No.5, Mar 1950)	Adjuvant	0.75	0	1.0	2.25							Died Week 4	
A83	As for A82	Adjuvant	2.0	0	0	2.86	3.375	3.35	4.0		3.84	4.06		126

* *Pool Nos. 7 and 8.*
† *See footnote Table II.*

TABLE V

GROUP D MONKEYS: RESPONSE TO REPEATED INTRAMUSCULAR INJECTIONS OF LANSING VIRUS

Monkey No.	Vaccine suspended in	Course of vaccination: Weeks 0, 2, 4, 6, 8, 11 13 — Amount of cord,* gm.	Week 0	2	4	6	8	11	13	Outcome of experiment	Total volume of immune serum collected, ml.
			Serum titers†								
A147	Water, all doses	1.0	0	0	2.7	3.025				Died after bleeding Week 6	21
A148		1.5	0	1.53	2.25	3.33	4.0	4.0		Died after bleeding Week 11	60
A150		1.75	0	1.83	2.0	3.0	2.95	4.0	4.05		36
A152		1.75	0	1.5	<1.0	2.25	2.76	4.0	3.64		
A155	Adjuvant first dose, water for subsequent	1.75	0	0	0	1.9	3.5	4.15	4.0		48
A156		1.25	0	2.15	2.5	3.35	3.15			Died in Week 8	42
A158		1.75	0	2.63	3.64	3.7	4.36	4.14	3.875		50
A166	Adjuvant, all doses	1.0	0	3.337	4.38	5.0				Died after bleeding Week 6	69
A167		1.75	0	0	3.25	4.0	3.775	4.63	4.77		102
A168		1.75	0	0	1.375	3.0	3.0	4.0	5.0		81
A170		1.75	0	1.78	In progress	3.64	4.7	4.63	5.5	Died after bleeding Week 13	69

* *Pool Nos. 7 and 8.* † *See footnote Table II.*

Group D

This group originally contained 28 monkeys. None of the monkeys had been used experimentally before the commencement of the vaccination program. The animals were divided into three lots, one of which received virus

TABLE VI

CUMULATIVE ANTIBODY TITERS IN MONKEYS RECEIVING DIFFERENT INOCULA

Inoculum	Monkeys represented	Cumulative antibody titers at following weeks					
		2	4	6	8	11	13
Water for all doses	A147, A148, A150, A152	1.2	2.13	3.05	3.17	4.04	3.7
Adjuvant for first dose, water for subsequent	A155, A156, A158	2.15	2.63	3.55	3.48	4.25	3.9
Adjuvant for all doses	A166, A167, A168, A170	1.7	3.02	3.775	3.65	4.5	4.95

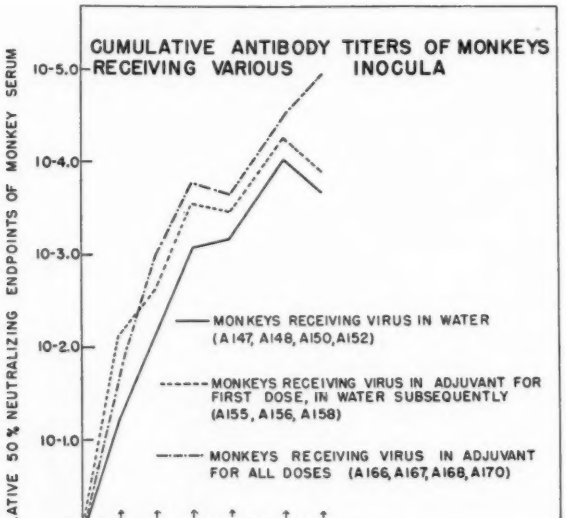
CUMULATIVE ANTIBODY TITERS OF MONKEYS RECEIVING VARIOUS INOCULA
CUMULATIVE 50 % NEUTRALIZING ENDPOINTS OF MONKEY SERUM
10-5.0
10-4.0
10-3.0
10-2.0
10-1.0
0
MONKEYS RECEIVING VIRUS IN WATER (A147, A148, A150, A152)
MONKEYS RECEIVING VIRUS IN ADJUVANT FOR FIRST DOSE, IN WATER SUBSEQUENTLY (A155, A156, A158)
MONKEYS RECEIVING VIRUS IN ADJUVANT FOR ALL DOSES (A166, A167, A168, A170)
1 2 3 4 5 6 7 8 9 10 11 12 13 WEEKS
(0.25 GM. OF CORD INOCULATED AT INTERVALS MARKED WITH ARROWS)

FIG. 2. Cumulative antibody titers of monkeys receiving various inocula.

suspended in water; one received the first dose of virus in adjuvant and subsequent doses in water; and the third lot was injected with virus in adjuvant on all occasions. The monkeys were not in good general condition, and no

less than 17 died early, after only one to three injections; four of these deaths were due to poliomyelitis, and the remaining deaths were attributed to trauma and blood loss in weak animals. In Table V are shown the details of the 11 monkeys that received four or more injections at intervals of two weeks. Because of the weak state of many of the monkeys, only comparatively small amounts of blood were withdrawn. Antibody levels of 3.0 were reached in most of the monkeys after three injections.

This experiment was originally designed to yield data as to whether antibody levels were higher in the animals receiving virus in adjuvant as compared to water. To aid in arriving at a conclusion on this point, cumulative antibody titers have been calculated for the monkeys in each of the three groups and these are shown in Table VI, and plotted in Fig. 2.

Each antibody titer was calculated from the sum of the mortalities of mice inoculated with various dilutions of serum from individual monkeys in the three groups.

Inspection of the data shows that the cumulative titers of the sera of monkeys receiving virus in adjuvant on one or more occasions are consistently higher than those of monkeys receiving virus in water. Further observations beyond Week 13 are needed to see whether the apparent differences in titer continue.

Pathological Changes in Vaccinated Monkeys

Practically all animals succumbing during the above vaccination programs were examined histologically. In no case were any lesions suggestive of acute or chronic poliomyelitis found in the central nervous system of monkeys that survived several immunizing injections. Four deaths from poliomyelitis occurred in Group D in monkeys that received one or two injections intramuscularly. It is evident therefore that even when repeated injections of live virus are given intramuscularly, the central nervous system does not appear to be invaded by the virus. It is of considerable interest that no evidence of "allergic encephalomyelitis" was found in any vaccinated animal.

No definite pathological changes were found in many of the animals which died. In some cases, death was probably due to withdrawal of a relatively large quantity of blood. Other animals were noted to become weak and somewhat oedematous during the progress of the vaccination program. This state was probably occasioned by the frequent loss of blood.

Tuberculosis presented somewhat of a problem during this investigation. Many of the animals that had been vaccinated for several months died of widespread tuberculosis. Early in January 1951, immediately on completion of the vaccination programs, the 13 surviving monkeys were tuberculin tested by the inoculation of 0.1 ml. of OT diluted 1 : 100 in the upper eyelids. The results were as follows:

Tuberculin test negative: A150, A152, A158, A167, A168.

Tuberculin test positive: 808, 809, 810, 812, A83, A116, A250, A155.

The animals giving a negative reaction had been in the animal house for just over three months. Most of those giving a positive reaction had been

in the colony for a much longer period, some for about a year. Monkey No. A155 however had only been in the animal house for somewhat over three months.

A number of strains of tubercle bacilli isolated from monkeys are at present being typed by Dr. Milton H. Brown, and this information will throw valuable light on the epidemiology of monkey tuberculosis.

Discussion

The object of this work was to produce sufficient quantities of immune monkey serum to allow of fractionation studies. This object was readily realized, as about three liters were obtained from the 30 monkeys discussed in this report. Our observations confirm those of earlier workers in the field with respect to the development of antibody in the serum following the intramuscular injection of Lansing poliomyelitis virus, although many titers are higher than those previously recorded. For example, several serum samples showed 50% neutralization end points at a $10^{-5.0}$ dilution of serum.

The general character of the antibody response to repeated injections of Lansing virus is well shown in Fig. 1, where the antibody titers of monkeys Nos. 810 and 815 are charted. These monkeys received three injections each of 0.25 gm. of infected cord intramuscularly in Weeks 0, 2, and 4. No virus was again given until Week 13, when a course of further fortnightly injections was commenced. It will be seen that antibody titers rapidly reached the 3.0 level. Antibody titers were maintained during the nine week rest period, and thereafter remained high.

This type of response was general throughout the 30 monkeys, although there were of course some variations. For example, the monkeys in Group A injected initially with a single large dose (1.0 gm.) of infected cord showed a much slower development of antibody. Levels at three and five weeks were mostly below 3.0. At 10 weeks, levels in the animals receiving virus in water were still below 3.0, but in those receiving virus in adjuvant the titers varied from 3.36 to 4.775.

It is of interest to compare the antibody responses in those monkeys receiving virus suspended in water with the responses in those vaccinated with virus incorporated in adjuvant. The experiment represented by the monkeys in Group D was specifically designed to answer this question. As shown in Fig. 2, antibody levels during a course of injections lasting for 13 weeks were consistently higher in the monkeys receiving virus in adjuvant on one or on all occasions than in the monkeys receiving virus in water. The differences in titer were, however, slight. In the monkeys in Group A that received a single large dose of virus there was likewise found evidence that at a certain stage (10 weeks) serum antibody levels were higher in those receiving virus in adjuvant.

Nevertheless, it is probable that individual monkeys vary in their response, irrespective of the antigen used, and certain monkeys vaccinated with virus in water, as for example No. 810 (Fig. 1), responded quite as well as any

monkey inoculated with virus in adjuvant (for example No. 815). It appears justifiable to conclude that in general the incorporation of infected cord in adjuvant has an enhancing effect on antigenicity as shown by serum antibody levels.

The method of immunization described served to furnish the large quantities of immune serum needed for preliminary fractionation studies. In the event that further quantities of immune serum are needed it is probable that a better method would be to inject the monkeys at intervals of two weeks for not more than three months and bleed at frequent intervals. This procedure, combined with monthly tuberculin testing should ensure an adequate supply of poliomyelitis immune serum from nontuberculous monkeys.

Acknowledgments

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We are particularly grateful to Drs. Robert Ward and Jules Freund for their advice and help throughout this work.

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THE NATURE OF THE CYTOTOXIC ACTION OF UNDISSOCIATED IODOACETIC ACID IN YEAST CELLS[1]

By J. G. Aldous[2]

Abstract

In order to determine the mechanism of the inhibition produced by undissociated iodoacetic acid, intact cells of baker's yeast were exposed to a 1.5×10^{-3} *M* solution of the inhibitor at pH 4.5. After washing and drying the cells, various enzyme solutions were prepared from them and their activities determined. Zymase, carboxylase, and catalase, as well as lactic and alcohol dehydrogenases were almost totally inactivated by exposure to the inhibitor. Evidence, at least with respect to zymase, indicates that the inhibition arises as a result of an inactivation of the protein components of the system. Thus the mechanism of action of undissociated iodoacetic acid appears to differ in several respects from that characteristic of the iodoacetyl ion whereas the ionic form of the acid produces an inhibition which is freely reversible and is more specifically associated with an inactivation of sulphohydryl groups, the inhibition resulting from the action of the molecular species is irreversible and nonspecific in nature.

Introduction

In an earlier communication from this laboratory (1) it was shown that the toxicity of monoiodoacetic acid to yeast cells, measured as a decrease in population viability, was a function of the concentration of undissociated acid in the cells' environment. This acid is entirely dissociated at pH values more alkaline than 5.5 and consequently the irreversible effect which characterizes its action in very acid solutions diminishes as pH 5.5 is approached and is entirely absent at neutrality.

The mechanism of action of iodoacetate has been amply studied over the past two decades, but since this work has been carried out at pH values close to 7.0 it must of necessity be a description of the mode of action of the iodoacetyl ion. The present investigation is therefore concerned with elucidating the manner in which the undissociated acid molecules produce their irreversible inhibition of cellular activity (respiration and cell division).

Instead of approaching the problem in the classical manner, i.e. by extracting various enzymes from the cell and exposing them to the inhibitory agent, a more direct method has been used. This involves exposing the intact cells to the inhibitor, extracting the various enzyme systems from them, and subsequently determining their activities. This technique presents two advantages: (*a*) the final terms of reference are always the intracellular rather than the extracted enzymes (*b*) it meets the often raised objection that in exposing an isolated enzyme to a given concentration of inhibitor one may be exposing it to a concentration many times that normally reached inside the cell. It should also be noted that use of the classical method referred to above is prohibited by the fact that in order to obtain an appreciable concentration of undissociated iodoacetic acid the medium must be buffered at about pH 4.5;

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Contribution from the Department of Pharmacology, Dalhousie University, Halifax, N.S.
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at this pH the enzymes under investigation are barely active and poorly soluble, and consequently evaluation of their activity must be made at pH values ranging from 6 to 7.2.

This method of determining the mechanism of action of an inhibitor is applicable to any agent as long as the effects upon the cell are irreversible. As in all studies where enzyme action is evaluated it is important to choose a proper base line to which the activity of various preparations may be referred. This base line is most conveniently taken as the activity (in arbitrary units) per mgm. of nitrogen contained in the sample of enzyme. Such an expression of activity, hereafter referred to as the "specific activity", has been used in the present investigation where there is a very real possibility that reduced enzyme activity could have resulted from a failure to extract the enzyme.

As a result of comparing the specific activities of five enzyme solutions prepared from treated and untreated samples of baker's yeast, it may be concluded that the inhibition produced by 1.5×10^{-3} *M* iodoacetic acid is a non-specific one, since each of the enzymes suffered an almost total loss of activity.

Materials and Methods

Preparation of Yeast Powders

(*a*) *Control Sample*

Two cakes of Fleischman's yeast (28 gm. each) were crumbled into 200 ml. distilled water and were freed from "filler" by repeated centrifuging and washing with distilled water. After decanting the supernatant from the final centrifuging, the cell paste was spread as a thin layer on the inside of a large (10 in.) pyrex basin, and compressed air (20° C.) was run in slowly overnight. The yeast was then allowed to dry at room temperature for about 40 hr., at which time the flakes of dry material were ground in a Wiley mill (No. 20 and finally No. 40 screens). The resulting powder was stored at room temperature in a tightly stoppered brown bottle.

(*b*) *Iodoacetate Treated Sample*

Two cakes of Fleischman's yeast were freed from "filler" as in the previous section and the resulting cells (46.5 ml.) were suspended in 100 ml. McIlvaine's citric acid – phosphate buffer (4) at pH 4.5. Twenty-three grams of glucose was added and the volume was brought to 400 ml. with buffer. One aliquot of this suspension (383 ml.) was placed in a 500 ml. florence flask and allowed to equilibrate for one-half hour at 20° C. with mechanical stirring. Without interrupting the stirring, 67 ml. freshly prepared iodoacetic acid (10^{-2} *M*, pH 4.5) was added and the mixing was continued for a further 30 min. The above quantities were chosen so that the final concentrations obtained would be: yeast 10%, glucose 5%, iodoacetic acid 1.5×10^{-3} *M*. The cells were then centrifuged, resuspended in distilled water, and recentrifuged. The cell paste was dried, ground, and stored exactly as in the previous section.

As a check upon the viability of the treated suspension aliquots were serially diluted (before the drying procedure) and mixed with Miller gelatin

medium (12). The control count was obtained by serial dilution of a 3.83 ml. aliquot (from the original 400 ml. stock suspension) to which had been added 0.67 ml. buffer. Viable cell counts showed 238 colonies for the control and 49 for the treated sample, or a viability of 21%.

Preparation of Zymase Fractions

Lebedew preparations from both the control and the treated powders were made according to a method adapted from that of Neuberg and Lustig (14). One gram of the dried powder was mixed with 1 or 2 gm. powdered Pyrex glass (Corning Glass Works first screen 20 mesh, second screen 35 mesh) and the dry mixture was ground in a heavy mortar for 20 min. at room temperature. This mixture was then extracted with 6 ml. *M*/15 ammonium phosphate, pH 7.93 for three hours at 37° C. with continuous stirring. The glass and cell debris were then centrifuged off and the supernatant liquid was stored overnight at 5° C. Next morning the extract was centrifuged for 10 min. and used in the Warburg vessels without dilution. This preparation will be referred to hereafter as the "apoenzyme".

The coenzyme fraction was prepared from each sample by thoroughly mixing 0.25 gm. of the dried cell powder with 2.5 ml. distilled water and placing in a boiling water bath for exactly three minutes. The coagulated protein was centrifuged off and the pale straw-colored solution was used without further dilution.

The commercially available barium hexosediphosphate was converted to the potassium salt for use in the experiments.

Carbon dioxide production was measured with the standard Barcroft–Warburg apparatus at 20° C. In establishing anaerobic conditions in the respirometers, tank nitrogen was freed from oxygen by passing it over freshly reduced copper gauze at 400° C.

Preparation of Carboxylase

Carboxylase was prepared by a slight modification of the method of Green *et al.* (8). One gram of yeast powder was mixed with 2 gm. powdered Pyrex glass and the dry mixture was ground at room temperature for 15 min. Ten milliliters of phosphate buffer pH 7.2 were added and the mixture was incubated at 37° C. for 16 hr.

Upon completion of the incubation period, 5 ml. distilled water were added and the glass and cell debris centrifuged off. To the supernatant fluid, 1.6 ml. *M*/2 phosphate buffer and 0.8 ml. molar calcium acetate were added. The white flocculent precipitate was centrifuged down and washed with 1.5 volumes of distilled water. The washings were then combined with the supernatant fluid from the previous centrifugation. To each milliliter of extract 0.38 gm. ammonium sulphate was added. A very fine precipitate formed which was almost completely thrown down by 45 min. centrifugation. The supernatant liquid was discarded and the precipitate was taken up in 3 ml. 0.04 *M* citrate buffer pH 6.0. The enzyme was again precipitated by the addition of 0.38 gm. ammonium sulphate per milliliter of solution and

centrifuged for 10 min. After being taken up in 3 ml. 0.04 *M* citrate the enzyme was stored in precipitated form under half-saturated ammonium sulphate (0.38 gm. per ml.). When used in the respirometers, the precipitated enzyme was pipetted directly into the vessels containing the citrate buffer.

Carbon dioxide production was measured in the standard Barcroft–Warburg apparatus at 20° C. Prior to their being placed in the constant temperature bath, all vessels were thoroughly flushed with oxygen-free nitrogen. Pyruvate was added to the enzyme solution from the side arm of the vessel following the initial pressure reading. The rates of reaction were computed from the initial slopes of a plot of carbon dioxide production against time.

Preparation of Lactic Dehydrogenase

For the preparation of lactic dehydrogenase the method of Ogston and Green (15) was adopted.

The original method was modified to the following: 1 gm. yeast powder was added to 2 gm. powdered Pyrex glass and the dry mixture was ground for 15 min. Following the addition of 10 ml. of 0.9% sterile saline the preparation was incubated overnight at 37° C. Next morning the glass and cell debris were centrifuged off. To every milliliter of supernatant fluid, 3 ml. saturated ammonium sulphate was added and the mixture was allowed to stand at 5° C. for one hour. The flocculent precipitate was then spun down and was finally taken up in 5 ml. of *M*/15 phosphate buffer pH 7.2.

The oxidizing power of the enzyme preparation was measured by the Thunberg technique at 20° C., the times for complete decoloration being taken as a measure of the activity.

Preparation of Alcohol Dehydrogenase

This enzyme was prepared according to the method of Still (16), which is similar to the procedure used by Dixon (6).

One gram of yeast powder was mixed with 2 gm. Pyrex glass plus 4 ml. distilled water, and was ground for 15 min. The cells and debris were then centrifuged down. The supernatant fluid was heated to 55° C. on a water bath and was maintained at that temperature for three minutes. Following this treatment the sample was stored at 5° for one hour and then centrifuged for 60 min. The enzyme was finally precipitated with half-saturated ammonium sulphate and taken up in phosphate buffer after centrifuging.

Determinations of activity were made utilizing the Thunberg methylene blue reduction technique. Here it was found necessary to add coenzyme I since the extraction procedure removes most of this material. Activities were calculated as in the previous section.

Preparation of Catalase

Yeast powder and Pyrex glass were mixed and ground as in the above procedures. Ten milliliters of distilled water was added and the mixture was allowed to stand at 5° C. for 15 hr. Glass and cell debris were removed by centrifugation for 20 min. For use in activity determinations it was necessary

to dilute the preparation 1 : 100. The total amount of oxygen evolved from hydrogen peroxide during the first six minutes of the reaction was used as the criterion of activity and was measured in the conventional Warburg vessel. Blank experiments using boiled enzyme solution indicated that no appreciable correction was needed.

Nitrogen Determinations

The nitrogen content of each enzyme preparation was estimated by the micro-Kjeldahl distillation procedure (11).

In all cases 0.5 ml. of enzyme solution was analyzed but where ammonium sulphate had been used in preparing the enzyme it was necessary to free the preparation of this material. To accomplish this 2 ml. of cold 95% ethyl alcohol was added to the 0.5 ml. aliquot of enzyme. The precipitate was centrifuged down, after storing 24 hr. at 5° C. and was then resuspended in 80% alcohol, cooled for one hour at 5° C., and recentrifuged. Following these washing procedures the coagulated protein was transferred to the digestion flasks.

Initial precipitation with six volumes of cold 10% trichloroacetic acid instead of alcohol yielded low nitrogen values for the precipitated protein.

Calculation of Specific Activities

As mentioned earlier, the specific activity of a preparation is given by the ratio of the activity to the nitrogen content of a given aliquot of enzyme solution. Since the units of activity often differ from one enzyme to another, it is desirable to express the specific activities in a manner which eliminates these differences and which thus brings them to a common basis for comparison. This may be most conveniently accomplished by expressing the activity of the iodoacetic acid treated enzyme preparation as a percentage of the control activity. When this value is divided by the nitrogen content of the treated sample, expressed as a percentage of the control nitrogen content, one obtains a fraction which may be readily translated into per cent control specific activity.

At least four preparations of zymase, carboxylase, and lactic dehydrogenase were made from both the control and the iodoacetic acid treated cells; two such preparations of alcohol dehydrogenase and catalase were made. The values reported in the results to follow are the averaged activities and the averaged nitrogen contents of these preparations. The standard deviations (S.D.) of the latter have been calculated and are shown in parenthesis following the average nitrogen content.

In order to obviate the possibility that storage, even in the dry state might be accompanied by a deterioration of enzymatic activity, preparations of any one enzyme solution from control and treated cells were always carried out concurrently.

Results and Discussion

The data in Fig. 1 depict the activity of one of a number of zymase preparations. Early experiments in this series established the fact that the zymase system prepared from iodoacetate treated yeast was entirely devoid of fermentative activity. The averaged nitrogen contents of the treated preparations was 0.354 mgm. (S.D. ± 0.053) while that of the controls was 0.646

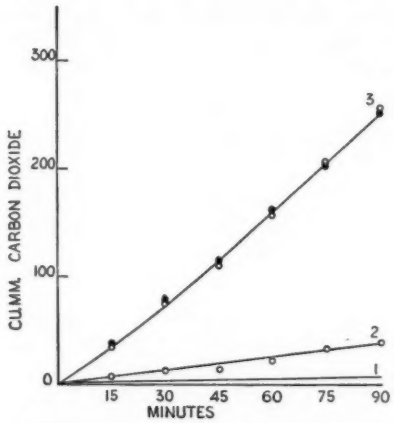

FIG. 1. The zymase activity of control and iodoacetic acid treated yeast. Each vessel received 0.5 ml. apoenzyme and 0.1 ml. coenzyme. Where added, the final concentrations of reagents were: glucose 3%, HDP (potassium salt) 3.2×10^{-3} *M*. Volumes were brought to a total of 2 ml. with *M*/15 $(NH_4)_2HPO_4$, pH 6.2. Gas phase: nitrogen; temperature 20° C. Nitrogen content of enzyme aliquots: control 0.625 mgm.; treated 0.348 mgm.

Legend: *Curve 1.* All mixtures containing treated apoenzyme with or without substrate, with treated or control coenzyme. *Curve 2.* ○ = Control apoenzyme + control coenzyme, no glucose or HDP. *Curve 3.* ○ = Control apoenzyme + control coenzyme + HDP + glucose. ● = Control apoenzyme + treated coenzyme + HDP + glucose.

mgm. (S.D. ± 0.054). Thus although the treated samples contained only 56% of the nitrogen of the control preparations the lack of activity in the former gives a specific activity of zero while that of the controls is 1.0.

By virtue of the complete system being composed of two fractions (see Methods) it was possible to determine whether the inhibition was specifically associated with the apoenzyme or with the coenzyme component. Accordingly these fractions prepared from both control and treated powders were mixed in various combinations.

The absence of any appreciable activity in those mixtures containing the treated apoenzyme is shown by Curve 1, while the rate of carbon dioxide production of the control samples in the absence of added substrate is indicated by Curve 2. Most important however is the fact that when the *control apoenzyme* is mixed with the *treated coenzyme*, the rate of carbon dioxide production is identical with that of the mixture containing both control components (Curve 3). There is thus little doubt that the undissociated iodoacetic

acid leaves the nonprotein part of the zymase system unaffected. According to Dixon (6) an analogous situation arises with respect to the action of the iodoacetyl ion. He found that short exposures to iodoacetate produced a much greater inactivation of the apoenzyme of yeast alcohol dehydrogenase than of the coenzyme when these components were exposed separately to the inhibitor.

Typical results from a determination of the activity of carboxylase preparations are shown in Fig. 2. Curves 1 and 2 show the activities of the treated

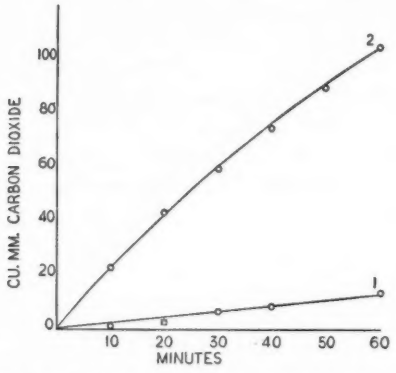

FIG. 2. The carboxylase activity of control and iodoacetic acid treated yeast. Each vessel contained the following reagents: Main space, 0.5 ml. enzyme, 1.2 ml. 0.66 *M* citrate buffer, pH 6.0; side arm, 0.3 ml. molar pyruvic acid, pH 6.0. Gas phase, nitrogen; temperature 20° C. Nitrogen contents of enzyme aliquots: control, 0.192 mgm.; treated, 0.100 mgm.

Legend: *Curve 1.* Iodoacetic acid treated sample. *Curve 2.* Control sample.

and of the control preparations respectively. Blank determinations, made either by omitting the enzyme or the pyruvate were negative. A computation of the rates of carbon dioxide production from the slopes of the curves during their initial portions, leads to the following values: control (Curve 2) = 3.0; treated (Curve 1) = 0.22. Taking into account the nitrogen contents of the extracted enzymes the specific activities indicate a value of 0.13 in this particular experiment. The nitrogen determinations for all control preparations of this enzyme averaged 0.122 mgm. (S.D. $\pm$ 0.029) while that of the treated preparations was 0.054 mgm. (S.D. $\pm$ 0.011). Since the enzymatic activity of all preparations made from the treated cells averaged 6% of that of the controls, an average specific activity of 0.13 is indicated, or an inhibition of 87%.

Evidence from the literature reporting the sensitivity of carboxylase toward iodoacetate is confusing. Barron and Singer (2) found that 5×10^{-3} *M* iodoacetate produced a 46% inhibition of carbon dioxide production. In contrast to this, Green *et al.* (9) state that their preparation (brewer's ale yeast) was unaffected by concentrations as high as 1.55×10^{-2} *M*. The present results would seem to indicate a more potent action for the undissociated acid.

A marked difference in the action of the ion and the undissociated acid is evident from the results of the lactic dehydrogenase determinations. In his search for an enzyme in yeast which would show a sensitivity to low concentrations of iodoacetate, Dixon (6) noted that the activity of extracted lactic dehydrogenase was reduced only 7% by a concentration of 10^{-2} *M*. He therefore concluded that this enzyme could not be involved in the reduced metabolic activity of the cell when the latter were exposed to concentrations of the order of 10^{-4} *M*.

The activity of one of four preparations of lactic dehydrogenase is shown in Table I. Other experiments indicated that the enzyme was fairly substrate-

TABLE I

THE ACTIVITY OF LACTIC DEHYDROGENASE PREPARED FROM CONTROL AND IODOACETIC ACID TREATED YEAST

Concentration of reagents added: lactate, *M*/10 in *M*/15 phosphate buffer pH 7.2; hexosediphosphate (potassium salt), 3.2×10^{-2} *M*, pH 7.2; coenzyme I (Schwarz), 10 mgm. per ml. in phosphate buffer; methylene blue, 1/5000 in phosphate buffer. Temperature 20° C. Nitrogen contents of enzyme aliquots: control, 4.42 mgm.; treated, 3.98 mgm.

Enzyme ml.	Substrate, ml.	Coenzyme I, ml.	Buffer, ml.	Methylene blue, ml.	Reduction time, min.
1.0 control	1.0 lactate	0.5	1.5	1.0	4¾
1.0 "	1.0 HDP	0.5	1.5	1.0	70
1.0 treated	1.0 lactate	0.5	1.5	1.0	130
1.0 "	1.0 HDP	0.5	1.5	1.0	α

specific, for, while a very slow reduction of methylene blue took place when hexosediphosphate was substituted for lactate, (see Table I) no reduction was noted with glucose, pyruvate, or alcohol. The averaged reducing activity of all treated samples gave a value of only 6% of the averaged control rate. The nitrogen contents of the control and treated samples were almost identical, being 4.45 mgm. (S.D. $\pm$ 0.042) for the former and 4.40 mgm. (S.D. $\pm$ 0.59) for the latter.

On the basis of a negative nitroprusside test, lactic dehydrogenase has been reported to lack sulphohydryl groups (10), and although such a test may indeed be negative when performed on a protein in its native state, the reaction usually becomes positive following the partial or complete denaturation of the protein (13). Thus, rather than indicating a lack of SH groups, a negative nitroprusside test may mean that the SH groups are not "exposed" to the reagent in the native state.

Early investigations (5) have shown that the inhibition by the iodoacetyl ion very definitely involves the sulphohydryl groups of enzymes. The observations of Dixon (6) on the relative resistance of this enzyme to iodoacetyl inhibition might therefore be conceivably related to its negative nitroprusside reaction, although evidence on this point is insufficient to permit this conclusion. The results of the present investigation show that no such problem

arises with respect to insensitivity of the enzyme toward the undissociated acid; the inactivation here is of the same order as that of the other enzymes studied. This observation, together with the fact that the inhibition is not reversible, suggests that some mechanism other than that characteristic of the iodoacetyl ion might be involved.

Dixon (6) reported that alcohol dehydrogenase suffered a far greater inactivation upon exposure to low concentrations of iodoacetate than did the other yeast enzymes he investigated. A solution of 3×10^{-4} *M* produced an inhibition of 92% which was almost entirely attributable to an inactivation of the apoenzyme. Our investigation of the zymase activity indicated that the coenzyme was not affected by exposure to iodoacetic acid and therefore it was deemed unnecessary to investigate the same phenomenon with respect to alcohol dehydrogenase. The results of a typical experiment in this series is shown in Table II, and from the nitrogen contents of these preparations one

TABLE II

THE ACTIVITY OF ALCOHOL DEHYDROGENASE PREPARED FROM CONTROL AND IODOACETIC ACID TREATED YEAST

Reagents used: ethyl alcohol 10%; coenzyme I (Schwarz) 1 mgm. per ml.; methylene blue 1/5000. All reagents dissolved in *M*/15 phosphate buffer, pH 7.2. Temperature 20° C. Nitrogen contents of enzyme aliquots: control, 0.858 mgm.; treated, 0.820 mgm.

Enzyme, ml.	Alcohol, ml.	Coenzyme I, ml.	Buffer, ml.	Methylene blue, ml.	Reduction time, min.
2.0 control	0.2	0.4	2.2	0.2	19
2.0 treated	0.2	0.4	2.2	0.2	120

obtains a specific activity of 0.16 for the treated sample. The averaged nitrogen contents of the treated preparations was slightly lower than that of the controls being 0.802 mgm. (S.D. ± 0.026) and 0.861 mgm. (S.D. ± 0.0056) respectively. The pooled specific activities of the treated preparations gave a value of 0.17, compared to that of 1.0 for the solutions of the untreated enzymes. This finding of as much as 17% of the control activity in the treated preparations may be accounted for by the fact that the population viability of the yeast from which this enzyme was prepared was 48% although the cells were exposed to the same concentration of inhibitor (1.5×10^{-3} *M*). One would therefore expect to see an even greater inhibition in a treated sample whose viability was only 20%.

An evaluation of the results from determinations of the catalase activity of the yeast powders (Table III) is difficult in the absence of data on the effect of the iodoacetyl ion with which to compare it. The specific activity of the iodoacetic acid treated sample was 0.06, showing an inhibition of 94%. Determinations of the nitrogen content of all preparations of catalase gave an average value of 2.34 mgm. (S.D. ± 0.231) for the control samples and 1.89

TABLE III

THE ACTIVITY OF CATALASE PREPARED FROM CONTROL AND IODOACETIC ACID TREATED YEAST

Reagents used: hydrogen peroxide 0.1 *M* in phosphate buffer; buffer, *M*/15 phosphate, pH 7.2. Temperature 20° C. Nitrogen contents of enzyme aliquots: control, 2.15 mgm.; treated, 2.08 mgm. (undiluted samples)

Enzyme, ml.	H_2O_2, ml.	Buffer, ml.	Activity, cu.mm. O_2/6 min.
0.5 control	0.3	1.2	240
0.5 treated	0.3	1.2	15

mgm. (S.D. $\pm$ 0.169) for the treated. As the enzymatic activity of the latter preparations was almost negligible, the data obtained from Table III provide a good representation of the degree of inhibition observed here. Data reporting analyses of catalase for the presence of SH groups do not unequivocally establish their absence (3). Thus it is impossible to decide whether this inactivation represents a mechanism distinct from that characteristic of the ion.

The results of the investigation as a whole suggest that in its undissociated form, iodoacetic acid, in low concentrations, acts as a nonspecific protoplasmic poison, i.e. one which affects many enzyme systems. However, before this can be definitely concluded, two other possible explanations must be considered. Firstly, as a result of adsorption phenomena, it is conceivable that the inhibitor might have been localized in discrete points within the cell. Secondly, the nonspecific action might have arisen by virtue of the internal environment of the cell being more acid than its external environment, since this would serve to decrease the dissociation of the inhibitor. Both these suggestions would tend to act in the same direction i.e., to increase the effective concentration of the iodoacetic acid so that its final concentration in the cell would be greater than that calculated from the environmental concentration. Within certain limits, the concentration of undissociated acid in the external environment may be altered in either of two ways: (*a*) by keeping the pH constant and varying the amount of iodoacetic acid added to the solution, or (*b*) by varying the pH of a solution containing a constant amount of the acid. In the paper referred to earlier (1) it was shown that regardless of which method was used to achieve a given concentration of undissociated acid, the physiological action (toxicity) was the same. Since this relation was observed to hold throughout the range of dissociation of the inhibitor, it follows that changes in the external environment must be accompanied by similar changes in the internal environment of the cell. This intracellular concentration of undissociated acid may be readily calculated. At pH 4.5 iodoacetic acid is about 70% dissociated, and since sufficient inhibitor was added to the cell suspension to make a 1.5×10^{-3} *M* solution, the concentration of undissociated acid must have been 4.5×10^{-4} *M*.

From the above discussion there is therefore good reason to believe that the results observed in the present investigation are not due to any inhibitor-concentrating action peculiar to the intracellular environment. The establishment of this nonspecific action of iodoacetic acid in acid solutions is an important one, for it emphasizes the influence that pH has upon specificity of action. That lowering of pH does cause a change in the character of the inhibition of yeast fermentation was noted many years ago by Ehrenfest (7) but it was not attributed to a reduction in the degree of dissociation of the inhibitor. It thus becomes clear that specificity of action is a characteristic of the iodoacetyl ion, and may only be expected to manifest itself in media more alkaline than pH 5.5.

Acknowledgment

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ACTION DE L'ACIDE ASCORBIQUE SUR LES EFFETS VASCULAIRES CARDIO-RÉNAUX DE L'ACÉTATE DE DÉSOXYCORTICOSTÉRONE (DCA)[1]

PAR OLIVIER HÉROUX[2] ET J. L. BONENFANT

Abstract

Earlier investigations have demonstrated that ascorbic acid prevents the hypertensive effect of DCA. Additional experiments on rats have shown that a combination of ascorbic acid and DCA results in stunted growth but does not prevent the renal hypertrophy or renal and vascular lesions usually caused by injection of DCA alone. It has also been observed that no cardiac or renal lesions occur in adrenalectonized DCA-treated rats. In normal animals, 17 days after treatment with DCA, vascular lesions are much larger and much more numerous in the heart than in the kidneys. Other workers have shown that cortisone in combination with DCA produces the same effects as those observed by the authors in animals treated with ascorbic acid and DCA. This similarity might be explained by assuming that ascorbic acid may act by stimulating the release of cortisone from the adrenal cortex.

Introduction

Friedman (1, 2) a démontré que chez les rats traités à l'acétate de désoxycorticostérone (DCA), des extraits lipidiques du cortex de la glande surrénale, donnés en fortes doses, préviennent les effets cardiovasculaires et rénaux de la DCA mais non l'hypertension, tandis que la cortisone produit l'effet contraire: elle inhibe l'action hypertensive de la DCA, sans prévenir ses effets sur le rein, le cœur et les vaisseaux.

Ayant déjà constaté que l'acide ascorbique à fortes doses reproduisait l'effet inhibiteur de la cortisone sur l'hypertension produite par injection de DCA (3), nous avons voulu savoir si le même phénomène se répétait dans le cas des effets vasculaires cardio-rénaux.

Nous avons à cet effet tracé des courbes de croissance, pesé le rein et les surrénales et finalement fait l'examen histologique du rein, du cœur et du pancréas chez des rats femelles d'un poids moyen de 135 grammes, employés au cours des expériences précédentes (3) et chez lesquels nous avions déjà constaté l'effet inhibiteur de l'acide ascorbique sur l'action hypertensive due à la DCA.

Protocole

Rappelons que tous les animaux furent néphrectomisés unilatéralement et reçurent comme eau de boisson du NaCl à 1% afin de les sensibiliser à l'action de la DCA.*

[1] *Manuscrit reçu le 13 août 1951.*
Contribution des départements de Physiologie expérimentale (Directeur, Dr. L. P. Dugal) et d'Anatomie pathologique, (Directeur, Dr. C. Auger) Faculté de Médecine, Université Laval, avec l'aide financière du Comité Médical du Conseil National de Recherches, Ottawa.

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* *Cette substance nous a été gracieusement fournie par la maison Ciba, Montréal.*

Les 141 animaux employés furent divisés en cinq groupes:

Groupe I: injection quotidienne sous-cutanée pendant 14 jours de 2.5 mg. de DCA en suspension dans l'eau (volume 0.1 cc.);

Groupe II: même traitement que le groupe I et en plus administration orale quotidienne de 160 mg. d'acide ascorbique sous forme d'ascorbate de sodium; quatre fois par jour les animaux étaient gavés avec 0.2 cc. d'une solution d'acide ascorbique à 20%;

Groupe III: même traitement que le groupe I, mais en plus les animaux de ce groupe ont subi une surrénalectomie bilatérale: une surrénale fut enlevée le premier jour de l'expérience et l'autre, le 10e jour;

Groupe IV: les animaux de ce groupe reçurent pendant 14 jours 160 mg. d'acide ascorbique sous forme d'ascorbate de sodium par voie orale, tout comme ceux du deuxième groupe, mais contrairement à ces derniers, ils n'ont pas été traités à la DCA;

Groupe V: témoins néphrectomisés mais ne recevant aucun traitement.

Dix-sept jours après le début de l'expérience, tous les animaux ont été sacrifiés par asphyxie à l'éther. Pour l'examen histologique, le rein, le cœur et le pancréas ont été fixés dans le Bouin, sectionnés à 5 μ et colorés à l'hémalun – erythrosine – safran.

Résultats

Poids du corps

Les sept premiers jours, l'acide ascorbique seul ou administré simultanément avec la DCA inhibe sensiblement la croissance (tableau I). Mais

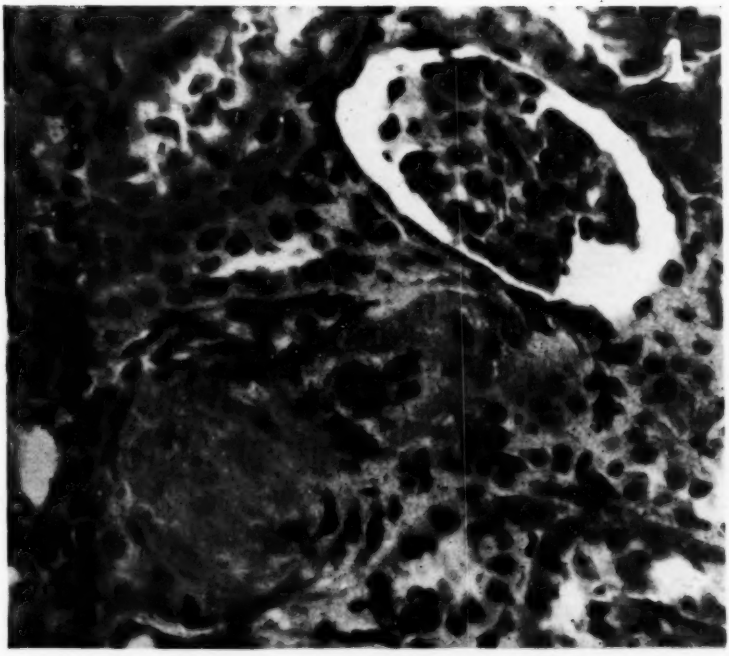
TABLEAU I

EFFET DE L'ACIDE ASCORBIQUE SUR LA CROISSANCE DES ANIMAUX TRAITÉS À LA DCA

Groupes	Poids initial, gm.	Poids après 7 jr., gm.	Poids après 14 jours, gm.	Augmentation de poids après 14 jr., gm.	Différence, gm.	*t*
I. DCA	131.6 (41)*	142.5	144.3	+ 12.7	+ 7.7	2.2
II. DCA + acide ascorbique	136.0 (31)	142.8	141.0	+ 5.0		
III. DCA + surrénalectomie	141.1 (19)	150.4	153.0	+ 11.9		
IV Acide ascorbique	133.7 (21)	135.1	145.5	+ 11.8		
V Témoins	139.5 (31)	153.8	157.9	+ 18.4		

* *Nombre d'animaux.*

PLATE I



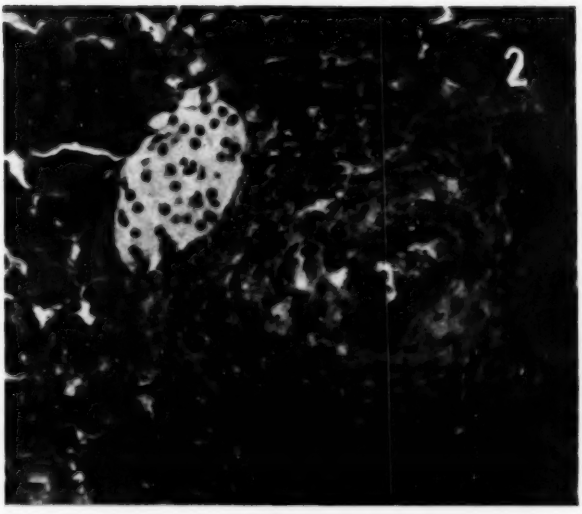


FIG. 1. Rein; groupe I traité à la DCA seulement. Coupe histologique montrant l'hyalinisation de la paroi d'une artériole préglomérulaire. ×525.

FIG. 2. Pancréas; groupe II traité simultanément à la DCA et à l'acide ascorbique. Coupe histologique montrant une dégénérescence d'une paroi artériolaire avec réaction fibroblastique périvasculaire et infiltration lymphocytaire. ×260.

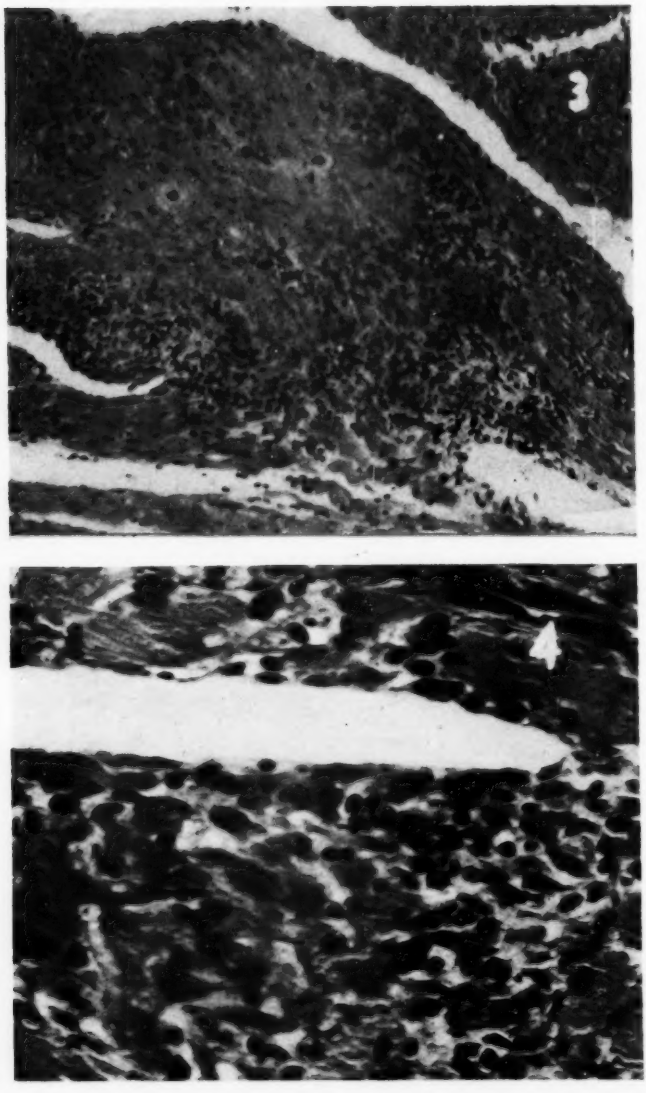


FIG. 3. Cœur; groupe II: DCA + acide ascorbique. Coupe histologique montrant un foyer intramyocardique de dégénérescence fibrinoïde avec réaction fibroblastique. ×130.

FIG. 4. Cœur; groupe I: DCA. Coupe histologique montrant une réaction fibroblastique péricapillaire. ×525.

entre le septième et le quatorzième jour, les animaux ne recevant que de l'acide ascorbique reprennent leur croissance, et le 14e jour ils manifestent une augmentation de poids semblable à celle des autres groupes traités. Chez les animaux traités simultanément avec la DCA et l'acide ascorbique, la croissance après 14 jours est sensiblement inférieure à celle des animaux traités seulement à la DCA. Comme ce résultat est statistiquement significatif, nous le considérons comme réel; cependant, le "*t*" étant plutôt faible, nous serions plus convaincu si une nouvelle expérience donnait le même résultat.

Poids des organes

Reins

L'acide ascorbique n'inhibe pas l'hypertrophie rénale causée par la DCA; administré seul, il n'a aucun effet sur le poids du rein, puisque chez les animaux recevant ce traitement le rein est du même poids que chez les témoins.

On peut noter aussi chez le groupe III, en l'absence de toutes autres hormones surrénaliennes, les animaux de ce groupe ayant été totalement surrénalectomisés, la DCA provoque une hypertrophie rénale beaucoup moins grande.

Surrénales

Dans tous les groupes le poids des surrénales, exprimé en mg. par 100 gm. de poids corporel, est le même (tableaux II et III).

Histologie

Reins

Chez les animaux surrénalectomisés traités à la DCA (groupe III) comme chez ceux traités à l'acide ascorbique seul (groupe IV), le rein est aussi normal que chez le groupe témoin (V); aucune lésion rénale n'y est observée.

Dans le premier groupe, le rein des animaux traités à la DCA seul manifeste les premières lésions apparaissant habituellement à la suite de l'administration de ce stéroide. Ces lésions consistent surtout en une légère hyalinisation de toute la paroi de quelques artérioles préglomérulaires (voir Fig. 1); dans un seul cas on a pu observer que la capsule de Bowman de quelques glomérules était recouverte d'un liséré hyalin. Parmi tous les animaux examinés, une proportion de 15.8% seulement manifestaient ces lésions artériolaires.

On a retrouvé la même proportion dans le groupe II recevant en même temps de la DCA et l'acide ascorbique; de plus les lésions étaient au même stage de développement, i.e., à leur tout début (voir tableau IV).

Cœur

Dans les trois derniers groupes, le cœur est parfaitement intact.

Chez les animaux traités à la DCA, c'est à ce niveau que nous observons les lésions les plus nombreuses et les plus avancées. Dans ce groupe, une proportion de 52.6% parmi les 19 animaux examinés manifestent les lésions intramyocardiques suivantes: manchon fibreux autour de quelques artérioles et flots de dégénérescence fibrinoïde avec réaction fibroblastique (voir Figs. 3 et 4).

TABLEAU II

EFFET DE L'ACIDE ASCORBIQUE SUR LE POIDS DU REIN ET DES SURRÉNALES CHEZ LES ANIMAUX TRAITÉS À LA DCA

Groupes		Rein, mg./100 gm.	Surrénales mg./100 gm.
I DCA	(15)*	1.020 ± 0.03	30.2
II DCA + acide ascorbique	(19)	0.980 ± 0.10	29.1
III DCA + surrénalectomie	(19)	0.890 ± 0.03	—
IV Acide ascorbique	(21)	0.660 ± 0.02	29.2
V Témoins	(31)	0.660 ± 0.02	29.1

* *Nombre d'animaux.*

TABLEAU III

ANALYSE STATISTIQUE DES RÉSULTATS DU TABLEAU PRÉCÉDENT

Groupes	Rein, mg./100 gm.	Différence	t
I. DCA	1.020 ± 0.03		
II. DCA + acide ascorbique	0.980 ± 0.10	+ 0.040	0.4
I. DCA	1.020 ± 0.03		
III. DCA + surrénalectomie	0.890 ± 0.03	+ 0.130	4.3
II. DCA + acide ascorbique	0.980 ± 0.03		
IV. Acide ascorbique	0.660 ± 0.02	+ 0.320	3.1
IV. Acide ascorbique	0.660 ± 0.02		
V. Témoins	0.660 ± 0.02	0.0	0.0
I. DCA	1.020 ± 0.03		
V. Témoins	0.660 ± 0.02	+ 0.380	10.5

TABLEAU IV

PROPORTION DANS CHAQUE GROUPE DES ANIMAUX MANIFESTANT LES LÉSIONS RÉNALES ET VASCULAIRES

Organes	I DCA, %	II DCA + acide ascorbique, %	III DCA + surrénalectomie, %	IV Acide ascorbique, %	V Témoins, %
Rein (19)*	15.8	15.8	0	0	0
Cœur (19)	52.6	58.3	0	0	0
Pancréas (10)	0	33.3	0	—	0

* *Nombre d'animaux.*

TABLEAU V

PROPORTION DANS CHAQUE GROUPE DES ANIMAUX MANIFESTANT DES LÉSIONS INFECTIEUSES

Organe	I DCA	II DCA + acide ascorbique	III DCA + surrénalectomie	IV Acide ascorbique	V Témoins
Pancréas	16.6% (10)*	0% (10)	50% (10)	—	0% (10)

* *Nombre d'animaux.*

Dans le groupe des animaux recevant en même temps de la DCA et de l'acide ascorbique, une proportion équivalente soit: 58.3% manifeste les mêmes lésions, mais il semble ici que ces lésions soient à un stage plus avancé.

Pancréas

Cet organe est intact dans tous les groupes excepté dans le 2e (DCA et acide ascorbique) où l'on peut noter dans un tiers des cas, une dégénérescence de la paroi de quelques artérioles, une réaction fibroblastique périvasculaire et assez souvent une infiltration lymphocytaire (voir Fig. 2).

De plus, nous avons relevé un fait assez intéressant en étudiant cet organe: chez les animaux traités à la DCA seule, dans 16·6% des cas on peut observer une pancréatite interstitielle se manifestant par une infiltration polynucléaire et plasmocytaire; chez les animaux surrénalectomisés traités à la DCA cette réaction inflammatoire apparaît dans 50% des cas alors qu'elle est tout à fait absente chez les animaux non surrénalectomisés traités simultanément à la DCA et à l'acide ascorbique (voir tableau V).

Discussion

Bien que l'acide ascorbique seul inhibe assez fortement la croissance durant les premiers jours de traitement, cet effet semble transitoire car au 14e jour, les animaux ainsi traités gagnent autant de poids que ceux des autres

groupes traités à la DCA. De plus l'acide ascorbique seul n'a aucune influence sur le poids du rein et des surrénales et ne cause aucune lésion rénale ou vasculaire.

Par contre, associé à la DCA, l'acide ascorbique semble inhiber la croissance même au 14e jour, n'empêche pas l'hypertrophie rénale, ni les lésions au rein, au cœur et au pancréas; en effet, chez les animaux traités à la DCA seule, comme chez ceux traités simultanément à la DCA et à l'acide ascorbique, l'hypertrophie rénale est du même ordre, les lésions rénales sont aussi avancées et se présentent chez autant de sujets; de même pour les lésions artériolaires au niveau du cœur. Au niveau du pancréas, cependant, la situation est un peu différente; l'acide ascorbique semble ici favoriser l'apparition plus hâtive des lésions artériolaires; en effet, 33·3% des animaux traités à la DCA et à l'acide ascorbique présentent des lésions pancréatiques et aucun n'en manifeste chez ceux traités seulement à la DCA.

Au cours de leurs expériences sur la DCA, c'est au niveau du rein que Masson (4) et Friedman (2) observent l'apparition des premières lésions vasculaires; après une vingtaine de jours de traitement à la DCA, ces auteurs ne remarquent aucune lésion au cœur des animaux ainsi traités. Or, au cours de notre expérience, après dix-sept jours de traitement seulement, nous avons noté au cœur, des lésions beaucoup plus avancées qu'au rein; les lésions rénales étaient très minimes et n'apparurent que dans 16·8% des cas tandis que les lésions cardiaques beaucoup plus graves apparurent dans plus de la moitié des cas, dans les deux groupes traités respectivement à la DCA et à la DCA plus acide ascorbique.

Associé à la DCA, l'acide ascorbique se comporte donc comme la cortisone puisque dans les mêmes circonstances ils donnent tous deux les mêmes résultats, à savoir, prévention de la croissance, aucune influence inhibitrice des effets de la DCA tels que: hypertrophie du rein et lésions au rein, au cœur et au pancréas, et comme nous l'avons vu dans des travaux publiés antérieurement: tous deux empêchent l'hypertension habituellement produite par la DCA.

A notre avis, ces similarités suggèrent fortement que l'acide ascorbique à fortes doses favorise une plus grande apparition de cortisone dans l'organisme soumis à un déséquilibre hormonal surrénalien, déséquilibre qui peut être occasionné soit par un "stress" soit par un apport exogène excessif de DCA.

Au cours de ces expériences, nous avons fait certaines observations secondaires qui semblent favoriser aussi l'hypothèse que nous venons d'émettre.

Chez 16% des animaux intacts traités à la DCA, on relève une pancréatite interstitielle, alors que 50% des animaux privés de leurs surrénales et traités de la même façon manifestent cette lésion inflammatoire; par ailleurs, si l'on administre de l'acide ascorbique aux animaux intacts traités à la DCA, aucun ne souffre de pancréatite. Il semble donc que chez les animaux intacts, la présence des glucocorticoïdes confère une certaine résistance aux infections provoquées par la DCA puisque, en l'absence de ces hormones chez les animaux surrénalectomisés, cette résistance diminue sensiblement. Comme

l'acide ascorbique par contre augmente cette résistance, on peut croire que cet effet de l'acide ascorbique est dû à ce qu'il provoque une plus grande production de ces glucocorticoïdes.

Une façon entre autres de vérifier cette dernière possibilité serait de voir si l'acide ascorbique associé à la DCA préviendrait encore l'apparition de ces lésions inflammatoires chez des rats surrénalectomisés.

Résumé

Nos expériences précédentes nous ont déjà montré que l'acide ascorbique inhibait l'action hypertensive de la DCA.

Dans les observations que nous venons de décrire, nous avons vu de plus que l'acide ascorbique associé à la DCA semble bloquer la croissance, ne prévient pas l'hypertrophie rénale ni les lésions rénales et vasculaires habituellement produites par l'administration de DCA seule.

Comme l'association de la cortisone à la DCA donne les mêmes résultats (2), nous émettons l'hypothèse que, chez les animaux traités à la DCA, l'acide ascorbique favorise une plus grande production ou une plus grande libération de cortisone dans le cortex surrénal.

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CONTRIBUTION ON THE BEHAVIOR AND STRUCTURE OF THE CYTOPLASMIC MEMBRANE OF BACTERIA[1]

By Roland Fischer[2] and P. Larose

Abstract

The relations previously found to exist between the bactericidal activity of substances and their affinity for wool have been used as the basis for a further study of the similarity between Gram-negative bacteria and intact wool on the one hand, and between Gram-positive bacteria and degraded wool on the other. A correspondence in behavior has been shown to exist in the case of Gram-staining, of the Eisenberg–Gutstein techniques, and of the Feulgen reaction. It was possible to produce with degraded wool a conversion from a Gram-positive state to a Gram-negative one (corresponding to that of intact wool) just as it is possible to carry out the similar conversion with Gram-positive bacteria by using oxidizing and/or acidic agents. Reconversion to the original Gram-positive state was also shown to be possible with reducing and/or alkaline agents. A mechanism is offered to explain the above findings and the respective cytochemical reactions characteristic of the cytoplasmic membrane of the bacteria. This mechanism is based on similarities in composition and structure (α-keratin) between the cytoplasmic membrane of Gram-positive bacteria and degraded wool, and the "cytoplasmic membrane" of Gram-negative bacteria and intact wool. Further possible similarities are pointed out. A new definition for bactericidal action is given in the light of these results. A direction for future work is indicated and discussed.

1. Bactericidal Action and Affinity for Wool

In previous communications (20, 21, 47) it was shown that there is apparently a correlation between the affinity of water-soluble substances for wool and their bactericidal activity* against Gram-positive and Gram-negative bacteria. This correlation was demonstrated for chemically different compounds of the anion-active and cation-active types.

A survey of previous work shows that in order to have a bactericidal effect, a compound must have a minimum affinity for wool.** Certain substances have been shown to have an affinity for wool, but their affinity for bacteria is apparently too low to enable them to exert a bactericidal action (47) against either Gram-positive or Gram-negative bacteria, such as *Staphylococcus aureus* and *Salmonella paratyphi* B. Examples for this behavior are given in Table I.

The action of cation-active substances differs from that of anion-active substances in a greater bactericidal activity towards Gram-positive bacteria for a given wool affinity, and also a more rapid increase of this activity with increase of wool affinity. The greater activity of cation-active (basic)

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**The term "bactericidal activity" as used throughout this paper is not meant to include bacteriostatic action.*

***Unless otherwise stated, whenever we mention wool we always refer to intact wool. What is meant by intact wool will be explained in the next section when other wools are being considered.*

TABLE I

RELATION BETWEEN WOOL AFFINITY AND BACTERICIDAL ACTION

Compound	Affinity for wool,* millimoles × 10^2 absorbed/gm. wool	Minimum bactericidal concentration* for *Staph. aur.* (Gram+)	Minimum bactericidal concentration* for *S. paratyphi* B (Gram−)
Anion-active			
1. Aerosol OT	2.0	0	0
2. Eulan new	5.0	1 : 240	0
3. Mitin FF pure	9.0	1 : 300	0
4. $HgCl_2$**	28.0	1 : 600	1 : 12800
Cation-active			
1. Sapamin KW conc.	2.8	1 : 800	0
2. Desogen	7.4	1 : 9600	1 : 1600

* *The affinity and the bactericidal activity are determined according to the methods previously described (21, 20).*

** *Although mercuric chloride is not an anion-active substance, it has been included here because it follows the order for anion active substances. However, it belongs to a class of metal salts whose action is probably different from that of anion active substances. This will be discussed later.*

substances towards Gram-positive bacteria might be explained on the basis of their greater affinity for the acidic Gram-positive bacteria. The bactericidal activity towards Gram-negative bacteria is apparently nonexistent for anion-active substances under our experimental conditions but it is present for the cation-active substances above a minimum threshold affinity value.

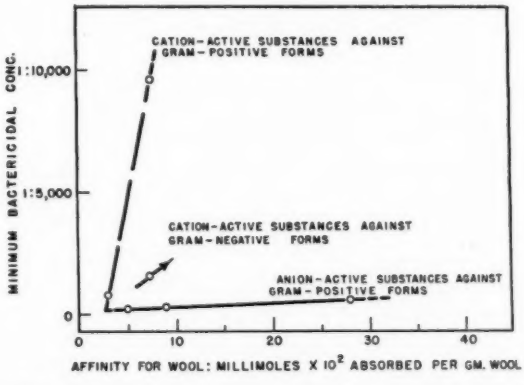

FIG. 1. Correlation between the affinity of substances for wool and their bactericidal properties.

This correlation between the affinity of various substances for wool and their bactericidal properties is schematically represented in Fig. 1, where we have attempted to show the connection.

There is another correlation between the affinity of these substances for wool and their bactericidal activity. It is known "that the anionic compounds affect only the Gram-positive species when tested at a neutral or slightly alkaline pH; Gram-negative organisms become susceptible to these substances at acid reactions. The cationic detergents are often more active and affect both Gram-positive and Gram-negative species over a wide range of pH although they are most active at alkaline reactions." (13, p. 281). It has also been shown that acidity favors the absorption of anionic substances by wool while the affinity for cationic substances is larger in alkaline solutions (20, 37). This correlation is also emphasized by the fact that increase of acidity also results in increased bactericidal effect. For example, McCulloch (39, p. 237) cites the increase in activity of benzoic acid and of salicyclic acid as the pH is lowered.

The connection between wool affinity and bactericidal action can be exemplified by numerous references in the literature. The work of Steinhardt *et al.* (50) shows that the affinity of acetic acid for wool is higher than that of hydrochloric acid. McCulloch (39, p. 235) quotes acetic acid as having a higher bactericidal action than hydrochloric acid. A similar comparison can be made between benzoic acid and monochloracetic acid. The latter has a lower affinity for wool (50) and according to Fabian and Bloom quoted by McCulloch (39, p. 242) benzoic acid inhibits the growth of microorganisms to a greater extent than monochloracetic acid does. Again, of various phenol derivatives tested by Steinhardt *et al.* (50) 2 : 4 : 6 trichlorophenol had the highest affinity for wool, while this compound is also the one with the highest bactericidal activity (39, p. 311) in a list of phenol derivatives given by McCulloch.

The relation between the bactericidal activity of a compound against Gram-negative bacteria and its activity against Gram-positive bacteria appears to be reflected by a similar relation between the affinity of the compound (as measured previously (22)) to intact wool and to degraded wool respectively. Intact wool has an isoelectric point (subsequently designated as I.P.) of pH 4.9 corresponding to that of the Gram-negative test organism, *S. paratyphi* B. When such wool is degraded with 0.15 *N* sodium carbonate solution at 80° C., its I.P. is lowered (16) and corresponds to the I.P.'s of Gram-positive bacteria.

This degradation consists of a hydrolysis of the —CO . NH— groups of the glutamine and asparagine of the wool protein, whereby ammonia is liberated and new carboxylic groups are formed, thus increasing the acidic character of the wool. Simultaneously, the wool loses 77% of its cystine content in four hours, but only 43% of its sulphur. After three–four hours, the curve for rate of decomposition becomes practically parallel to the time axis showing that the degradation is very slow* as shown by Fig. 2 which illustrates the course of the degradation according to Elöd, Nowotny, and Zahn (16). This is exemplified by the fact that the loss in cystine is only 6.7% of the original content between the four hour and 22 hr. degradations. Elöd, Nowotny,

* *The stability of the wool degraded for about two to four hours is reflected in the relatively constant values obtained in the affinity measurements (21).*

and Zahn (16) have indicated that, up to a four hour degradation, there is no appreciable change in the structure of the wool fiber under the microscope, but a 22 hr. degradation damages the wool visibly. The 15 to 45 min.

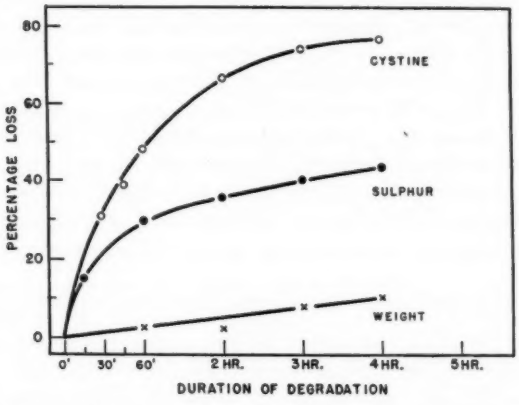

FIG. 2. Course of degradation of wool by 0.15 *N* sodium carbonate solution at 80° C.

degraded wool, whose degree of damage is much less than that of the wool treated for 85 min. to four hours, will be regarded as a medium damaged wool and will be referred to from now on as the M.D. wool, while the more highly degraded wool will be designated as the H.D. wool. The *intact wool* and the *H.D. wool* can be regarded as two stable types of α—keratin with I.P.'s corresponding to the Gram-negative and Gram-positive bacteria respectively and by comparing the affinity of a substance for these two wools, its relative bactericidal activity towards Gram-negative and Gram-positive bacteria may be predicted. Thus if a substance has a higher affinity for intact wool than for the H.D. wool, its bactericidal action against Gram-negative bacteria should be stronger than against Gram-positive bacteria and vice versa. Following the same reasoning it can also be concluded that substances that show little difference between their affinity for intact wool and for H.D. wool, should also show little difference in their bactericidal activity towards Gram-negative and Gram-positive bacteria* (21, 22).

In view of the above we may expect the Gram-variable bacteria to be represented by the M.D. wool since their behavior is intermediate between that of the Gram-negative bacteria and the Gram-positive bacteria, e.g. their I.P. (*Corynebacterium diphtheriae* 3.5 – 4.0**) lies between that for Gram-negative bacteria (4.8 – 5.8) and that for Gram-positive bacteria

** In the early work, wools degraded for 45 min. and for 86 min. respectively were taken as representing the behavior of the two types of bacteria. This was corrected to the intact wool and the two hour degraded wool in (22).*

*** These I.P. values are according to Stearn and Stearn (49) and are the basis for classifying this bacterium as Gram-variable. Bergey's Manual (1948, p. 584) lists this bacterium as Gram-positive but not intensely so in older cultures.*

(1.9 – 2.9) (13, p. 74). Gram-variable forms are generally more readily affected by bactericidal agents than are Gram-negative or Gram-positive bacteria; this should be indicated by higher affinity of the M.D. wool for these active substances since the M.D. wool is less stable than the intact wool or the H.D. wool. Affinity measurements for the M.D. wool will form the subject of a separate paper. It might be noted here that although only a few anion- and cation-active substances were compared for their bactericidal activity and their affinity for wool, these were chosen at random as representatives of different classes of active substances, and we have no reason to doubt that others would also show the correlations to which we have already referred. The bactericidal activity of many of such substances is well known (39, p. 362–366). The affinity of some of them for wool is also recognized (30), and the behavior discussed is not peculiar for the few substances chosen.

2. The Similarity Between the Cytoplasmic Membrane of Bacteria and Wool

After the correlations just mentioned were found, the question arose as to why the three wool proteins, namely intact wool, M.D. wool and H.D. wool, are related in respect to their affinity for various substances in such a way that they seem to reflect the bactericidal activity of the same substances against Gram-negative, Gram-variable, and Gram-positive bacteria respectively. Reference has already been made to the similarity between the I.P.'s of the above-mentioned wool proteins and those of the corresponding bacteria.

A further resemblance between the three wool proteins and the three types of bacteria is shown experimentally by applying the *Gram-staining* technique (27) for bacteria differentiation to the intact wool, the M.D. wool, and the H.D. wool.* The staining was carried out as follows: about 0.12 gm. of intact wool and the same weight of H.D. wool were treated at 45° C. for one minute with 1 ml. of a Gram gentian violet solution diluted with 10 ml. of water. The samples were then rinsed in water and treated again at 45° C. for one minute with 1 ml. of Gram iodine – potassium iodide solution and finally decolorized three times with 10 ml. portions of boiling 96% ethanol for a few seconds. The solutions used are those usually employed in bacteriology and contained 0.5% gentian violet with 0.012% phenol in the dye solution and 1.1% potassium iodide and 0.55% iodine in the "mordant" solution. One may use the original undiluted Gram staining solutions at room temperature, but the smaller amount of solution is not so convenient and the treatment has to be prolonged for three–four minutes as in bacteriology.

The intact wool is found to be colorless (Gram-negative) while the H.D. wool is stained a deep violet (Gram-positive) and the M.D. wool is found to be more or less deeply colored (Gram-variable).

In view of this further similarity in behavior between the three wool proteins and the three types of bacteria, one may ask how far can this comparison

* *The wool yarn with which the following tests were carried out is one which was extracted with ether and with alcohol and then distilled water, whereas the early experiments have been carried out with ordinary commercially washed wool.*

be extended because it is obvious that the complex bacterial bodies cannot be similar in all respects to the wool proteins.

It is well known from the results of Henry and Stacey (32*a*, 32*c*) that Gram-positive bacteria extracted with a solution of 2% sodium choleate lose gradually their positiveness and give a Gram-negative cytoskeleton. At the same time ribonucleic acid is liberated.

The same effect can be produced by the enzyme ribonuclease but this acts only on the dead surface-damaged Gram-positive bacteria (13, p. 82). The resistance of living Gram-positive cells against ribonuclease is probably due to the fact that the cell wall and particularly the cytoplasmic membrane are intact in the living organisms. That the change from Gram-positive to Gram-negative bacteria as carried out by Henry and Stacey refers to the "inner part" and not to the outer layers of Gram-positive organisms is conceivable because, firstly, normal and intact Gram-negative cells are not modified in their Gram-staining behavior by placing them in a neutral solution of the magnesium salt of pure yeast nucleic acid (13, p. 82) and secondly the Gram-negative cytoskeletons ("inner part") of formerly Gram-positive bacteria produced by the sodium choleate extraction can be restored by "replating" the magnesium salt of pure yeast nucleic acid back on them. Dubos expresses the same view (13, p. 81) when he states that "it is possible, by extraction with bile salts, to strip off the Gram-positive outer layer from a number of bacterial species, and to separate a Gram-negative skeleton". In an attempt to duplicate Henry and Stacey's *bile salt treatment* with the H.D. wool, since this wool so far has behaved like the Gram-positive bacteria, samples of the latter were treated for varying periods up to five weeks with bile salt, at 60° C. in physiological saline solution. As a result of this treatment, the wool did not become entirely Gram-negative in behavior. However, as the period of treatment became longer, it was noticed, on examination of the fibers under the microscope, that the staining ability of the outer part of the fibers is gradually lost. The treatment is apparently not sufficiently severe to convert the H.D. wool fully to a Gram-negative one. This is due, perhaps, to the thickness of the wool fibers which is relatively much larger than that of bacteria, and even more so, when compared with the cytoplasmic membrane, given as about 100 Å. (7, p. 19). These results show that although the three wool proteins are able to simulate, through their affinity, the bactericidal activity and the Gram-staining behavior of the three types of bacteria, the H.D. wool shows a different behavior from that of the Gram-positive bacteria when one tries to apply Henry and Stacey's technique. This is no doubt due to the fact that Henry and Stacey's replating technique is applied to extracted bacteria, whereas our wools simulate the behavior of unextracted bacteria only. It appears therefore that the behavior of the wool reflects only those characteristics of the bacteria which depend on the surface layer. It follows that the surface of the three types of bacteria must have some structural components similar to those present in the three types of wool. If this is so, the intact

and the H.D. wool proteins should give other reactions which are characteristic of the "ectoplasm" of the two extreme types of bacteria. But in the "ectoplasm" of bacteria, the strong affinity for dyes is limited to the cytoplasmic membrane for Gram-positive forms and "is one of the last structures to be decolorized in the Gram-negative forms", as pointed out by Knaysi (35). The cell wall of the "ectoplasm" plays no part in the reaction since it has little affinity for dyes (13, p. 37), so we can restrict ourselves to the characteristics of the cytoplasmic membrane. *The transformation of the Gram-positive bacteria into Gram-negative bacteria by acids* following Deussen (10) can be *reproduced with H.D. wool*, our model for Gram-positive bacteria. Not only can we convert the H.D. wool (Gram-positive) into one which simulates the behavior of Gram-negative bacteria by treatment with *N* Hydrochloric acid for three–four minutes at 50–55° C., but we can "replate" this Gram-negative wool (or an intact wool) to render it Gram-positive by treating it with a *reducing agent* such as sodium bisulphite or formaldehyde. It will be recalled that in their "control" experiments Henry and Stacey, during their bile salt extraction (32*a*, p. 394), found that washed cells rendered Gram-negative by the sodium choleate, could be reconverted to Gram-positive cells by treatment with formaldehyde.

The odor of hydrogen sulphide detected during the hydrochloric acid treatment of the H.D. wool indicates that the reconversion to the Gram-negative state cannot be carried on indefinitely, but only to the point where all the remaining sulphur is in the form of stable linkages at which time the wool will always show the Gram-negative behavior irrespective of any reducing action. This stage corresponds to the completion of the extraction with bile salt as carried out by Henry and Stacey (32*a*) who took as their end point of the extraction, the point at which the reconversion of the bacteria from Gram-negative to Gram-positive after reduction with formaldehyde was no longer possible.

Since the Gram staining solution has reducing properties, the conversion from Gram-negative to Gram-positive bacteria may also be carried out by a more severe treatment with the *gentian violet solution*, e.g., by prolonging the time to several minutes or by raising the temperature.*

Further similarities between the behavior of wool and that of bacteria can be cited. Both intact wool and *intact* Gram-negative bacteria can be degraded with alkali or by proteolytic enzymes (3, 24, 45). On the other hand, *intact* Gram-positive bacteria are resistant to alkaline treatment, as is the H.D. wool (3; 16; 45; 13, p. 83).

A cytochemical reaction pertinent to our discussion is that of Eisenberg–Gutstein (14, 29) which consists in treating a preparation of Gram-positive

* *The objection might be raised that the Gram-staining behavior is not peculiar to wool but that other proteins would also show the same analogy. However, it must be evident by now that the wool owes this behavior to some specific structural groups. The Gram-staining technique was tried on intact degummed silk. Its behavior was different from that of intact wool since it stained a deep violet.*

organism with tannin and then staining with a basic dye. The "*ectoplasm*" *alone** will take the stain. This technique was applied to the intact wool and to the H.D. wool, 0.12 gm. of each being treated with 10 ml. of 5% tannin solution for 1 min. at 45–50° C. and then stained with 1 ml. of Gram gentian violet solution, diluted with 10 ml. of water as before, at the same temperature for one minute. This was followed by boiling three times for a few seconds with 10 ml. of 96% ethanol. (Other basic dyes such as basic fuchsin can be used instead of the gentian violet.) This procedure results in a dark coloration of the H.D. wool but leaves the intact wool colorless. This is further example of the H.D. wool behaving as the cytoplasmic membrane of Gram-positive bacteria. The comparison between the behavior of the intact wool and that of the Gram-negative bacteria is only partial because when Gram-negative organisms are similarly treated "the whole cellular body is uniformly stained, either slightly or deeply, according to the species, the dye and time of treatment". (13, p. 83).

> By applying the procedure to intact wool yarn tied into a knot, it is found that the knotted portion of the yarn is colored like the H.D. wool, whereas the remaining portion of the yarn is left colorless. The straining of the wool fibers caused by the knot results in a breakage or weakening of the S—S bonds (24) which renders the wool more susceptible to degradation during the 45–50° C. tanning and dyeing treatment. Consequently, the more the fibers are stained, the greater the number of bonds broken and the more readily is the wool degraded. This is reflected by a more intense coloration at the points of high strain.

A modification of the tannin treatment of *Eisenberg and Gutstein* (14, 29) gives results which show again the similarity in behavior between the cytoplasmic membrane of the Gram-positive bacteria and the H.D. wool. This consists in treating the wool or bacteria with a basic dye, then with tannin, and finally counterstaining with another basic dye of contrasting color. The body of Gram-positive bacteria is stained with the first dye and the cytoplasmic membrane with the second (13, p. 83). The H.D. wool which so far has been shown to behave like the cytoplasmic membrane of Gram-positive bacteria, when similarly treated, is stained with the second dye as expected. The Gram-negative bacteria treated in the same way are stained lightly or deeply with the second dye only. Our intact wool is stained very lightly with the second dye only.

Although the above staining experiments were carried out with dyestuffs which served to show visually the difference in affinity between the Gram-negative bacteria and Gram-positive bacteria, or between the intact wool and the H.D. wool, similar differences in affinity, which has nothing to do with the color of the absorbed substance (36), have been shown to exist with anion-active and cation-active substances in the first section.

* *A survey of the literature shows that there is still much confusion on the nature of the ectoplasm of bacteria. This may be illustrated by the following statement extracted from a paper by Bartholomew, J. W. and Mittwer, T. (2); "Unfortunately the nature of this area is not known and this has resulted in much confusion as to the meaning of this word when applied to bacterial cells. It is an area, which is chemically differentiated by the staining method used (Gutstein), but the reason for this differentiation is unknown".*

We conclude therefore that the mechanism of the reaction responsible for the differentiation and correlations found in the case of the staining reactions is probably the same as that for the correlations found between the bactericidal activity (affinity) towards Gram-positive bacteria and Gram-negative bacteria* and their affinity for the H.D. wool and the intact wool respectively. With this in mind, we can now explain, by means of such a mechanism, certain behaviors of the Gram-positive bacteria because we now know that the cytoplasmic membrane of Gram-positive bacteria, or part of it, must have some common chemical structural features with the H.D. wool or at least to one of the products of the degradation. It should be emphasized here that wherever we are referring to the similarity in behavior between the cytoplasmic membrane of the two types of bacteria and the two wool proteins, we have in mind only those reactive parts, which may be certain amino acid residues or a type of bonding, involved in the particular behavior under study.

Before discussing the mechanism of the Gram staining and Eisenberg–Gutstein staining reactions, the behavior of the intact wool and the M.D. and H.D. wools towards the Feulgen (17, 18, 54) reagent will be described, because Pennington (42) has observed a positive Feulgen reaction on the *surface*, as well as in the protoplast, of Gram-positive and Gram-variable bacteria after they had been treated with sodium metaperiodate under mild conditions.

Many of the investigators who have tried the Feulgen test on Gram-positive bacteria have failed to obtain a positive reaction on the bacterial surface because they had used hydrochloric acid to hydrolyze the desoxyribonucleic acid in the bacterial body in order to liberate the aldyhyde group sought. We are of the opinion that the failure to obtain a positive response on the surface was due to a strong oxidation occurring during the hydrochloric acid treatment to which we shall refer later.

The test** was repeated with and without sodium metaperiodate pretreatment on intact and H.D. wools and without the periodate pretreatment on wools subjected to intermediate degradation. The intact wool was colorless while the H.D. wool was definitely colored. The wools degraded for various times with sodium carbonate showed various degrees of coloration. The intensity of the color increased with duration of the degradation up to about 30–45 min., then decreased with increasing time of degradation until a fairly constant coloration was obtained after the two hours degradation. With

* *Tests carried out since the preparation of this paper and to be published later have indicated that the affinity relations found with the Gram-positive and the Gram-negative test organisms mentioned above can be extended to other bacteria and seem to be therefore fairly general.*

** *Feulgen reaction:*

About 0.1 gm. wool sample was placed in 10 ml. of the Feulgen reagent (without any prior hydrochloric acid treatment) and heated to about 50° C. for ½–¾ min. The wool was then washed with 45% acetic acid at 50–60° C. three times (¼ min.). This washing procedure was repeated, using acetic acid and ethanol solution (equal volumes) and finally boiled in 96% ethanol for a few seconds. The differentiation between the "true" and "pseudo" reactions was carried out according to Oster, K. A. and Mulinos, M. G. (J. Pharmacol. Exptl. Therap. 80 : 132. 1944) on the basis that the purple color developed in the former can be decolorized with dilute sodium hydroxide and restored to its original intensity with hydrochloric acid, while the reddish color of the latter cannot be restored by acid after the decolorization. Our positive Feulgen reaction in the case of the degraded wools is based on the above technique and is a "true" one.

periodate pretreatment according to Pennington (42), similar results are obtained except that the intensity versus time of degradation curve is displaced along the time axis towards shorter time of degradation. In other words, the coloration for any one time of degradation is more intense.

3. Mechanism of the Color Reactions

(*a*). *Gram Staining*

As a result of Henry and Stacey's experiments (32), one generally had in mind, when speaking of Gram-positiveness, a property associated with the presence of ribonucleic acid. It was generally believed that this property was associated with the cytoplasmic membrane, the "ectoplasm", or part of it. However, the Gram-positive character must be dependent also on the inner part, the "cytoskeleton" of Henry and Stacey, because it is possible to restore a Gram-positive behavior to this part of the cell by "replating" it with magnesium ribonucleate after reduction of the cell skeleton. Therefore, the inner part of the bacteria, as well as the cytoplasmic membrane, possesses a Gram-positive character. It should be possible to produce on this membrane a change from Gram-positive to Gram-negative behavior and vice versa by a reversible acid-base process which may also involve reduction and oxidation provided such actions do not result in an autolytic degradation of the cell. This claim is based on the similar behavior between the H.D. wool and the cytoplasmic membrane in the "control" experiments of Henry and Stacey (32*a*, p. 394).

The Gram-negativeness of Gram-negative bacteria is analogous to the Gram-positiveness of the Gram-positive bacteria in that it is associated with the inner part as well as with the outer layer. This is readily shown by the Eisenberg and Gutstein (14, 29) technique which indicates that the outer layer (membrane) allows the dye to penetrate to the inner part. Therefore, the absence of color in the inner part of the cell in the application of the Gram-staining technique necessarily indicates that the inner part of the cell is also Gram-negative.

The respective roles played by the ectoplasm and the protoplast in the Gram-positive staining reaction have been misunderstood. This is largely due to the undue stress laid on the ability of ribonucleic acid to "replate" the Gram-negative cytoskeleton resulting from the bile salt extraction (inner part of the bacterium) and to restore its Gram-positiveness. The confusion arises when this restoring power is also associated with the cytoplasmic membrane which cannot play a part in the restoration of the Gram-positive behavior, since it has been irreversibly destroyed.

In our opinion, it should be possible to perform the replating with substances less complex than ribonucleic acid. There is no indication in the literature that such replating with various acidic substances (with an acidity and combining capacity comparable to that of the ribonucleic acid) has been tried. The uncertainty regarding the Gram-positiveness as the behavior of a single complex is well shown by several statements found in the literature but the following will serve as examples: "The essential Gram-positive material must be located

in the outer layer of the protoplasm. However, the ectoplasm staining material is not in itself responsible for the Gram-positive complex (2, p. 43) or "There are other observations which also tend to show that Gram-positiveness depends upon the intactness of the cell wall" (13, p. 79).

We have already associated the behavior of H.D. wool and intact wool with that of the cytoplasmic membrane of Gram-positive bacteria and Gram-negative bacteria. In proposing a mechanism for the Gram-staining, we are limiting ourselves then to the behavior of the outer layer of the bacteria, but we do not imply that the mechanism for the behavior of the inner part of the bacteria is different but only that experimental difficulties limit our knowledge of the Gram staining behavior to the outer layer of the bacteria since any known procedure for removing the outer layer necessarily causes alteration of the original structure of the inner part.

We have seen that Deussen (10) could convert Gram-positive bacteria into Gram-negative bacteria with hydrochloric acid. This conversion can also be carried out with a variety of reagents (46) and the change has been shown to be reversible (42*b*). Henry and Stacey (32*b*) also report that *Streptococcus salivarius* which is Gram-positive becomes Gram-negative when grown in minimal amounts of magnesium or on acid media. This effect could be reproduced with our H.D. wool (Gram-positive) by treatment with hydrochloric acid, sulphuric acid, or acetic acid which rendered the wool Gram-negative. As for bacteria this conversion is reversible, because when the Gram-negative behaving wool is treated with alkalies, e.g. sodium carbonate, sodium hydroxide, ammonium hydroxide, the wool becomes Gram-positive. The same effect can be obtained with other substances that are not generally considered as converting agents or having strong alkaline reactivity. We have produced the change from the Gram-negative behaving wool into a Gram-positive one not only with formaldehyde and sodium bisulphite but with gentian violet itself by prolonging the time of the staining technique and/or raising the temperature.* In connection with the work of Polonovski and Cotoni (43) who found that "Sapamine MS", a quaternary base, could change pneumococcus I, which is Gram-positive into a Gram-negative form, we were able to convert our Gram-positive behaving 86 min. degraded wool into a Gram-negative one by treating it, e.g. with a 1 : 250 diluted "Sapamine KW conc." (another quaternary base) solution in water** for 10 min. at about 70° C.

The action of alkalies and acids on wool has been studied by many, but it will suffice here to refer to the work of Stoves (52) on the reactivity of the cystine linkage, as his experimental conditions approximate more closely to those of our alkaline degradation. The action of alkalies on wool is believed to be, at first, one of hydrolysis:

$$R.S.—S.R. + H_2O \longrightarrow R.SH + R.SOH.$$

* *We have succeeded in converting Bacillus cereus and Escherichia coli to the opposite Gram-staining behavior and reconverting them to the original Gram state by using the substances mentioned above. These experiments will be reported in a subsequent paper.*

** *This solution has a definite acidic reaction probably because the commercial product contains an excess of acid.*

The sulphenic acid formed, however, is unstable, and decomposes to give hydrogen sulphide and an aldehyde- or keto-acid.

Writing $R'CH_2SOH$ for R . SOH

$$R'CH_2SOH \longrightarrow R'CHO + H_2S.$$

The aldehyde group and the sulphydryl groups can then react in various ways to produce stable $-\overset{|}{\underset{|}{C}}-S-\overset{|}{\underset{|}{C}}-$ linkages.

Reducing agents also attack the disulphide bond of wool, giving rise to sulhydryl groups or derivatives, e.g.

$$\begin{aligned} R.S.\text{–}S.R. + NaHSO_3 &\longrightarrow R\,.\,SNa + R\,.\,S{-}SO_3H \\ \text{or} &\longrightarrow R\,.\,SH + R\,.\,S{-}SO_3Na. \end{aligned}$$

The rebuilding of cystine linkages by the union of two R . SH groups can result from the action of various reagents which act as oxidizers, e.g., potassium bichromate, acetic acid.

The changes produced by these various reagents and their correlation with the Gram-negative character of intact wool and the Gram-positiveness of the H.D. wool can be represented schematically as in Fig. 3.

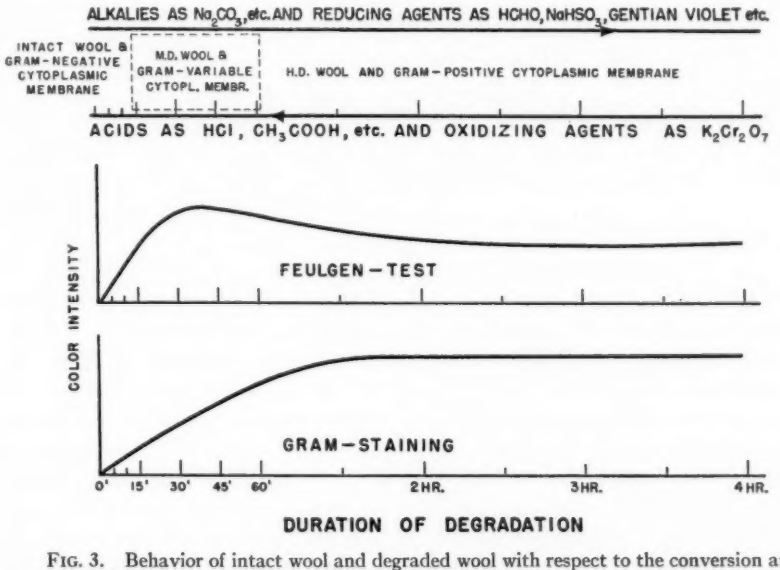
ALKALIES AS Na2CO3, etc. AND REDUCING AGENTS AS HCHO, NaHSO3, GENTIAN VIOLET etc.
INTACT WOOL & GRAM-NEGATIVE CYTOPLASMIC MEMBRANE
M.D. WOOL & GRAM-VARIABLE CYTOPL. MEMBR.
H.D. WOOL AND GRAM-POSITIVE CYTOPLASMIC MEMBRANE
ACIDS AS HCl, CH3COOH, etc. AND OXIDIZING AGENTS AS K2Cr2O7
COLOR INTENSITY
FEULGEN-TEST
GRAM-STAINING
0' 15' 30' 45' 60' 2HR. 3HR. 4HR.
DURATION OF DEGRADATION

FIG. 3. Behavior of intact wool and degraded wool with respect to the conversion and reconversion by means of various agents, the Feulgen reaction, and Gram-staining.

Having pointed out the action of oxidizing agents (or acids) in stabilizing the sulphur linkages, we can now offer an explanation for the Gram-staining behavior of bacteria including the role of mild oxidizing agents such as picric

acid, potassium bichromate, etc. (13, p. 75). We have seen that the dye itself (gentian violet) has some reducing as well as basic property and may shift the Gram-negative character towards the Gram-variable stage. It will not affect the Gram-positive cytoplasmic membrane since this is already in the reduced state. After treatment with the dye solution, the Gram-positive bacteria, because of their acidic character, are stained by the basic dye, which immobilizes the reactive groups of the membrane, while the relatively basic Gram-negative membrane is practically unstained. However, the adsorption of dyestuff molecules during the staining operation reduces to a small extent the Gram-negative membrane towards the Gram-variable state and the membrane becomes slightly stained. The application of mildly oxidizing agents following the staining procedure will rebuild the reduced groups of the Gram-negative membrane (but will not affect the immobilized groups of the Gram-positive membrane) thus making the differentiation between Gram-negative bacteria and Gram-positive bacteria sharper. The action of the subsequent alcohol "decolorizer" is to extract the uncombined dye from the bacteria. This is not a true decolorizing action since the alcohol does not destroy any color.*

Up to now, our discussion has been limited to the similarity between the Gram-positive bacteria and the H.D. wool on the one hand, and between the Gram-negative bacteria and the intact wool on the other, as they represent two extreme types. However, similarities in behavior also exist between the Gram-variable type (e.g. in respect to *C. diphtheriae*) and M.D. wool. The similar behavior is not limited to the correlations already given in the first section but can be extended to all the staining reactions which have been applied to the intact wool and the degraded wools. Even when the technique of straining the wool by knot formation is applied to the wools being tested, e.g. by the Feulgen reaction, or by the Gram staining, it is found that the intensity of color of the knots is shifted in the direction of the H.D. wool.

The position of the Gram-variable bacteria appears to be one intermediate between that of Gram-negative bacteria and that of Gram-positive bacteria, because the degree of oxidation or reduction, as well as its acidity, lies between the two extreme types.

In this connection, it may be cited that Dubos (13, p. 84) has already suggested that the differences between Gram-positive and Gram-negative species under a variety of conditions are of a quantitative rather than of a qualitative nature.

There have been reports that the Gram-variable behavior can be altered as a result of a sort of mutation (13, p. 84). This change may be connected with

** The counterstaining frequently used has nothing to do with this mechanism. It consists in a physical adsorption of the dye by the decolorized Gram-negative bacteria while the Gram-positive organism is prevented from picking up more dye because of the previous combination with the gentian violet or other dyestuff used. The sorption of the dye by the Gram-negative bacteria in the counterstaining may be facilitated by the increased acidic character of the bacteria resulting from the oxidizing treatment with iodine (13, p. 75).*

the oxidation–reduction mechanism offered. If the Gram-staining response depends on the degree of oxidation or reduction of the bacterial "ectoplasm", then the variations in the intensity of staining given by certain strains are readily understandable.

(*b*). *Eisenberg–Gutstein Differentiating Staining*

The technique which involves treatment with tannin followed by staining with a basic dye can be explained as follows. In a strongly acidic medium such as we have in Gram-positive bacteria, the irreversible fixation effect of tannin on proteins—producing a "leather"— is the highest (28). When this "leather" is treated with basic dyestuffs, it is stained, but it is not permeable to the dye; hence, the body of the Gram-positive bacteria is not affected by the dyestuff. The lack of permeability of the "leather" for basic dyestuffs also explains the second test in which a tannin treatment followed instead of preceded the basic dye first applied. In this case, the second dye is prevented from penetrating through the "leathered" cytoplasmic membrane but not the first dye.

In Gram-negative bacteria (or with the intact wool) on the other hand we have a minimum "leathering" effect (28) in view of the higher I.P. of these bacteria but the tannin has also a reducing action and acts on the Gram-negative bacteria in the same way as the other reducing agents already mentioned in explaining the Gram-staining mechanism. The Gram-negative bacteria acquire, therefore, the property of attracting basic dyes. In the second procedure of Eisenberg and Gutstein, the bacteria are reduced by the tannin only after treatment with the first basic dye. They can therefore combine only with the second dye.

(*c*). *Feulgen Reaction*

We have pointed out that the alkaline degradation of wool gives rise to reducing groups but that at the same time a recombination takes place giving rise to stable linkages. It follows that during the early stages of degradation, the number of reducing groups will increase but this will be followed by a decrease as a result of recombination. We readily understand now the change of intensity of the color to Schiff's fuchsin obtained in the Feulgen test when applied to wool of various degrees of degradation and why we obtain a maximum in the color which, under our experimental conditions, correspond to a wool degraded for 15–30 min. This is represented by the lower curve in Fig. 3. It should be recalled that our Feulgen test was carried out without pretreatment with hydrochloric acid.

If the H.D. wool is treated with hydrochloric acid, the Feulgen positive reaction is obtained only after prolonged treatment with the reagent, or after reduction with bisulphite. In view of the known action of hydrochloric acid

on the H.D. wool (see Fig. 3), it is obvious that it will oppose the action of the Feulgen reagent. The development of the color is no doubt due to the fact that the Feulgen reagent itself has some reducing power and will, in the long run, counterbalance the effect of the hydrochloric acid. This opposing effect of the hydrochloric acid, which has been used by many bacteriologists with the purpose of liberating deoxyribonucleic acid before applying the Feulgen test, accounts for the negative result obtained in such cases. However Knaysi (35*b*) obtained a positive Feulgen reaction on the cytoplasmic membrane of *B. cereus* and Pennington (42) who tried the test without the use of hydrochloric acid succeeded in obtaining a positive reaction with Gram-positive bacteria and Gram-variable bacteria. He remarked that the surface as well as certain parts of the interior were colored.

Bielig, Kausche and Haardick (6) have reported experiments which indicate that Gram-negative bacteria acquire a reducing property once the pH of the culture medium has reached the I.P. of the bacteria and this reducing property becomes more and more pronounced as the medium becomes more and more alkaline until the death rate becomes so great that the reducing power decreases again as a result of decreasing numbers. Unfortunately, these investigators did not apply any staining reaction that would indicate the gradual transition to the Gram-variable and then to the Gram-positive stages.

. . . . .

Our previous conclusions regarding the similarity in behavior of the cytoplasmic membrane of Gram-negative bacteria and of Gram-positive bacteria to that of intact wool and H.D. wool lead us to expect that other properties and characteristics of these two wool proteins are to be found in the cytoplasmic membrane of the two types of bacteria.

In Table II are listed a number of such properties pertaining to intact wool and to H.D. wool.

We mention in Table II that intact wool is degraded under the action of ultraviolet light to give a more acidic wool. No one appears to have followed the course of this reaction on wool which had already been degraded but since the chemical changes produced by light (attack of disulphide bonds) are analogous to those resulting from alkali degradation, one might also expect a slower action of the radiation on wool that had been partly degraded such as our H.D. wool. According to Gärtner (23) Gram-negative bacteria are destroyed about four times more rapidly than Gram-positive bacteria by the light from a mercury vapor lamp. This is in line with the behavior of wool just cited.

It is impossible here to give a general review of the properties of intact and degraded wool. For further information, the reader should refer to various periodicals. An excellent review of the recent views on the structure and composition of wool has been given by Lindley (38).

TABLE II

PROPERTIES OF H.D. WOOL COMPARED WITH INTACT WOOL

Property	Intact wool	H.D. wool
Cystine content	Ca. 11.7%* (16)	Ca. 2.9% (16)
Nitrogen content	Ca. 15.5% (16)	Ca. 14.8% (16)
Total sulphur	Ca. 3.9% (16)	Ca. 2.3% (16)
X-ray structure	α-Keratin with side chain spacing of 9.8 A. (16)	Same as for intact wool (16)
Isoelectric points	4.9 (31, 48, 15)	Lower than intact wool
Methyl green – pyronine** staining applied simultaneously on both wools	Reddish violet	Blue
Fehling test for reducing substances	Negative	Positive
Ammoniacal nitroprusside test for —SH groups	Negative (8)	Positive (8)
Ultraviolet light	Degradation producing acidic wool (9, 41)	. . . . .

* *The cystine content of wool varies between fairly wide limits but this figure is very close to the average value.*

** *Methyl green – pyronine test: About 0.1 gm. of H.D. wool was tied to about 1 gm. of intact wool (this preparation can be regarded as a rough model for the nucleus and the protoplasma of a cell). The combined wool sample was treated at 80° C. for a few minutes with 10 ml. of a methyl green – pyronine solution (Unna-Pappenheim standard mixture) diluted with 25 ml. of water, then rinsed in lukewarm water and dried. The H.D. wool was stained blue while the intact wool acquired a reddish violet color. This is a further indication that the H.D. wool is definitely more acid than the intact wool, since methyl green is more basic than pyronine and will be more strongly absorbed by the relatively more acidic H.D. wool.*

4. The Nature of the Cytoplasmic Membrane

At first, it may seem strange to the bacteriologist that the cytoplasmic membranes of Gram-negative bacteria and of Gram-positive bacteria apparently behave chemically like the intact wool and the H.D. wool respectively, which have normally an α-keratin structure. Bisset (7, p. 28) has shown that bacterial flagella are growing through the cell wall which is the outermost layer next to the cytoplasmic membrane. The flagella probably originate in the cytoplasmic membrane itself and are an outgrowth of it. On the other hand, X-ray examination of the flagella of *Bacillus subtilis* and of *Proteus vulgaris* by Astbury and Weibull (1) showed that they contain a protein belonging to the α-keratin–myosin–epidermis–fibrinogen group.

On the basis of these observations, the analogy in chemical behavior between the wool and the bacteria does not appear extraordinary.

It must be pointed out that the discovery of basic dyestuffs dates back to 1856 when Perkin first discovered mauvein. This made it possible for Gram (27) in 1884 to discover with another basic dyestuff (gentian violet) the staining properties of Gram-positive bacteria. There is no mystery attached to the differentiation of Gram-positive bacteria from Gram-negative bacteria by this staining technique since it has been known for a long time that basic dyestuffs have an affinity for acidic substances. It was also long known that methylene blue was active against the acidic Plasmodium of the malaria quartana. It was found much later (1911) by Hörlein, Dressel, and Kothe (33) that by adding a sulphonamide group to a dyestuff its affinity for wool was increased since its fastness to washing was greater than that of the dyestuff without the sulphonamide group. However, the full significance of these observations on the affinity of various substances for wool and their relation to their bactericidal properties was not realized since after the interregnum of the Prontosils it was only in 1935 that we have the first record of Domagk (11, p. 141) on the activity of sulphonamides (basic) against streptococci (acidic) but even then the relation between the affinity to wool and to bacteria was overlooked in spite of an obvious connection.

The process of flagella formation with an α-keratin structure, and probably that of the transverse septa themselves, from the globular protein inside the cell is quite possibly similar to the one which gives rise to the wool fiber from the follicle (40).

There are numerous references in the literature on the behavior of the cytoplasmic membrane as a semipermeable one (13, p. 32–34). This is not inconsistent with the view that it may be composed of a protein with an α-keratin structure.

5. Definition of Bactericidal Action

The foregoing results lead us to a re-examination of the nature of bactericidal action and to define it. There does not appear to be a precise definition of bactericidal action. Dubos in his excellent text on *The Bacterial Cell* (13) spends 15 pages in describing the bactericidal and bacteriostatic actions.

In the light of our results we can state that substances that are bactericidal towards Gram-negative, Gram-variable, and Gram-positive bacteria are either *anion-active* and *cation-active* substances which alter chemically and/or physically the cytoplasmic membrane of these bacteria, in such a way that the membrane is no longer capable of playing its role in the metabolism of bacteria, or *nonionic* substances which owe their bactericidal activity probably to their coagulating or denaturing action on the inner part of the bacterial body after having permeated the ectoplasm without necessarily changing its structure.

The anion- and cation-active substances probably act by blocking basic and acidic active groups respectively of the cytoplasmic membrane. The action of metallic salts, such as mercuric chloride, may result from the interaction between the mercury and the —S—S— bond of the cystine present in the membrane protein and this probably explains why the mercuric chloride is more active against the Gram-negative bacteria with comparatively intact

—S—S—bonds than against the Gram-positive bacteria where the sulphur appears to be in the reduced form such as —SH (see Stoves). The divalent mercury reacts more readily with the —S—S— link than with the —SH groups, because the two sulphurs are readily available, while in the case of the reduced —SH groups, most of these are too far removed from each other to enable the mercury to react with two of the groups at the same time. There may be substances with such a constitution that they may at the same time affect the —S—S— bond and either the acidic or the basic groups of the membrane.

As regards the action of nonionic substances, we may mention as example the bactericidal action of the lower alcohols or aldehydes which are free to pass through the outer cell wall and cytoplasmic membrane to denature the inner proteins of the bacterial body irreversibly.

So far, we have spoken of the affinity of various substances for wool and for bacteria only in a general way. However, the nature of the substances in the environmental conditions must be of importance and we may ask, at this point, what are some of the factors which control the affinity. Some of these factors are:

1. Whether the substance is an anion-active or cation-active one (i.e. acidic or basic in nature).

2. Nature of the environment, e.g. temperature, presence of salts, pH, etc. (12, 5, 25, 53).

3. The degree of dissociation of the substance. (The higher the degree of dissociation, the lower the affinity) (50, 44, 34).

4. The size and the molecular weight of the substance (51).

5. The chemical nature of the substance. The number and kind of reactive groups such as —OH, —SH, —NH_2, etc. must certainly influence its affinity. (19, p. 390).

6. Possible Application of the Similarity Between Wool and the Bacterial Cytoplasmic Membrane

As our knowledge in the chemical behavior of wool progresses, it will no doubt become possible to determine the groups that are responsible for the various reactions and correlations discussed before, and thus to predict the bactericidal behavior of various substances, natural and synthetic, from their composition. However, until then, in our opinion, the first consequence of practical importance of the findings discussed above would be to test a large number of agents for their bactericidal activity towards a wide range of representative bacteria on the one hand, and their affinity to intact wool, M.D. wool, and H. D. wool on the other. The results would then be tabulated in such a way as to permit a comparison of the activity with the affinity to the various wools and enable a general index to be assigned to each substance as an indication of its bactericidal activity under the particular conditions of the test in relation to its affinity to the wools. This would be followed by

an examination of the effect of different environmental conditions—pH, temperature, salt concentration, and various media including bacterial broths and blood sera of various animals as well as the individual blood proteins, etc.—on this index. If the change in environmental conditions for any one class of active substances and any one type of bacteria results in a similar change in the index, then the bactericidal activity of a new substance may be simply defined by a few affinity tests towards various wools. The effect of varying environmental factors on this activity will be determined by the index found for the particular substance under test when this is compared with indices of substances belonging to the same class.

The reason for including blood sera and blood proteins in particular amongst the environmental factors to be studied is that it is believed that an approach to a more exact chemotherapy with bactericidal agents will have been made when it will be possible to relate the bactericidal activities (affinities for wools) of a compound with its capacity to interact with a particular blood protein. There seems to be a connection between this interaction and the toxicity, blood level of the substance, and the mechanism of excretion (4). A good start towards this problem has been made and summarized in a review by Goldstein (25).

Conclusions

Intact wool and the more acidic highly degraded (H.D.) wool (α-keratins) apparently give many cytochemical reactions which are characteristic of the cytoplasmic membrane of Gram-negative bacteria and of Gram-positive bacteria respectively.

This similarity in behavior may make it possible to predict generally the bactericidal activity of a substance against Gram-negative bacterial and Gram-positive bacteria from affinity measurements of the substance for intact wool and H.D. wool respectively.

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THE ACTION OF LIPIDOLYTIC ENZYMES OF LARVAE OF *GALLERIA MELONELLA* ON VIRULENT *MYCOBACTERIUM TUBERCULOSIS*[1]

By Edith Mankiewicz

Abstract

Virulent *Mycobacterium tuberculosis* treated by lipidolytic enzymes of larvae of *Galleria mellonella* become less acid fast to Ziehl-Neelsen and auramine O staining. On the other hand, the enzyme treated bacilli are stained by Sudan black B and retain the dye after treatment by acetone. At the same time they appear to be thinner, and their granules heavier than before treatment. According to the concentration of the enzymes and the period of exposure, growth is arrested, or very much delayed. The delayed growth is smooth and spreading and the organisms retain the altered morphology and staining characters. Virulent tubercle bacilli treated by enzyme extracts give a negative neutral red cytochemical reaction and lose their virulence as judged by guinea pig inoculations. Enzyme treatment produces a slight increase in the streptomycin resistance of virulent tubercle bacilli.

Extracts from larvae of *Galleria melonella* (the wax moth) have been shown to possess a strong lipidolytic activity on lipid fractions extracted from tubercule bacilli (4). In the present study, after success was attained in increasing the concentration of the specific enzymes in these extracts by precipitation and lyophilization, suspensions of virulent bacilli from the H37Rv strain were exposed to the enzymes' action. This exposure resulted in changes in the staining properties of the bacilli, in their morphology, their cultural behavior, their cytochemical reaction to neutral red, their virulence for guinea pigs, and their streptomycin sensitivity.

The Preparation of the Enzyme Solution

The preparation of powder from the larvae, and of extracts from the powder, followed the technique detailed in our previous study (4), with the exception that the fresh larvae were disintegrated in a Waring Blendor. The buffered extracts were sterilized by filtration through "Zsigmondy Membranfilter No. 5" and the sterile filtrate was precipitated by Seitz filtered acetone at 5° C.; after the precipitate was washed with acetone, it was dissolved in phosphate buffer solution of pH 7.2, and reprecipitated several times. It was finally freed from acetone by vacuum, redissolved, distributed in ampules, and lyophilized to dryness. The resulting light brown powder showed no loss of activity in six months and readily dissolved in distilled water to any desired strength.

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Study of the Effect of Enzyme Solution on the Staining Properties and on the Morphology of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis of strain H37Rv from 10-day-cultures in Dubos medium containing Tween 80 were centrifuged, washed three times in saline, and suspended in enzyme solution to the same volume as the original culture. These suspensions with controls in heat inactivated enzyme were kept at 37° C. for the period of the experiment. While in the control the arrangement of the virulent bacilli in cords was maintained, in the active enzyme disintegration of the cords into single cells, or several lying side by side, was observed after two to three days exposure at 37° C. Throughout the 20 days of the experiment, the organisms in the control (inactivated enzyme solution) had the form and staining reactions of virulent tubercle bacilli in normal cultures; they were acid fast to 20 min. decolorization, negative to Sudan black B, and fluorescent with auramine O. After the suspensions of bacilli in active enzyme solution had been kept for one week at 37° C., the bacilli appeared to be thinner, and their granules heavier than those of the controls. At this time, the bacilli were still of normal acid fastness, and, like all culture grown organisms, they did not retain Sudan black B dye after decolorization by acetone. After 16 days' exposure, smears made from the enzyme suspension contain long and thin rods arranged in chains and their granules are distinct. These chains are not acid fast and do not retain auramine fluorescence after decolorization by acid alcohol. Stained by 1% osmic acid with "secondary staining" in alcohol the bacilli appeared black and faintly beaded, while the organisms from the controls are faintly and uniformly stained brown. Stained by Sudan black B according to the technique of Sheenab and Whitwell (6) or according to our modification of the technique using a Sudan black B solution in dioxane, with gentle steaming for five minutes instead of burning off the dye, 90% of the long and thin bacilli arranged in long chains retained the dye after treatment by acetone. Besides these elements arranged in chains, there are rods of normal aspect, only when they are stained by steaming carbolfuchsin, they are decolorized by 3% hydrochloric acid, 25% sulphuric acid, and 30% nitro-acid, if the time of decolorization exceeds three minutes.

Bacilli from these enzyme treated suspensions produced a particular type of growth on Lowenstein medium, which will be described later, but their arrangement and staining characters remained as seen in the active enzyme solution. Cultures from the controls gave normal cultures of virulent tubercle bacilli.

These results show that there is a change in morphology and a reduction in acid fastness as indicated by the time of resistance to decolorization, owing to the action of the lipidolytic enzymes of *Galleria melonella*. There is also a loss of auramine fluorescence and a more intense staining by osmic acid. The connection between loss of lipid material by treatment of tubercle bacilli with fat solvents and loss of acid fastness is still controversial, but according to Bekker and Tasman (1), auramine O is a "fat-dye" and the extraction of tubercle bacilli by fat solvents causes loss of auramine O fluorescence, with, at

the same time, loss of acid fastness. In their reaction to Sudan black B, osmic acid, and auramine O, the enzyme treated organisms resemble those found in pathological exudates, in contrast to culture organisms. They differ from organisms in both exudates and culture in their degree of Ziehl-Neelsen staining and morphology.

The affinity of Sudan black B dye to enzyme treated bacilli could also be demonstrated in a macroscopic cytochemical test. Bacillary suspensions in active and inactivated enzyme solutions were washed twice in saline after 16 days at 37° C. and suspended in 50% acetone in water. To each tube, some drops of a dilute solution of Sudan black B in 50% acetone in water was added. The cells in both tubes settle to the bottom. After five minutes, those of the suspension in active enzyme are progressively covered by a thin layer of dark blue dye while, at this time, no dye settles over the cells from the inactive enzyme solution. After 20 min., the thin dark layer of dye over the cells of the test is well formed, whereas, in the controls, the dye remains in solution for 45 – 60 min. after which time dye particles begin to sediment in the control.

These and other observations concerning the Sudan black affinity of enzyme treated bacilli suggest that the Sudan black fastness of tubercle bacilli in tuberculous animals is due to the disintegration of the bacillary lipids by enzymatic action. This is emphasized by the observation that in culture-grown organisms, the change from nonfastness to fastness to Sudan black could not be reproduced by extraction of the lipids by ordinary fat solvents.

Study of the Effect of Enzyme Solution on the Cultural Properties of *Mycobacterium tuberculosis*

The addition of enzyme solution to Dubos Tween medium inoculated with H37Rv resulted in a bacteriostatic action which is probably largely due to the hydrolysis of the Tween constituent of the medium by the enzymes, releasing oleic acid in bacteriostatic concentrations. Lipase contained in the albumen fraction No. 5 of the medium has been held responsible for a similar action (2).

The effect on multiplying bacilli in Proskauer-Beck medium varied according to the concentration and length of time of action of the enzymes added. It produced either complete bacteriostasis or a poor growth in the depth of the medium and absence of surface growth. In the experiments with Proskauer-Beck medium the bacteriostatic effect obtained is not due to the action of the enzymes on a constituent of the medium, but to that on the bacilli.

Instead of adding enzyme solutions to the growing liquid culture, tubercle bacilli from cultures in Dubos Tween medium or in Proskauer-Beck were washed in saline and suspended in the enzyme solution. After various periods of time of exposure at 37° C., the cells were washed in saline and subcultured on Lowenstein medium. According to the concentration of the enzymes in the suspensions, or to the time of exposure of the bacilli to the enzyme action, cultures from the suspensions gave either normal growth, or delayed growth with atypical colonies, or no growth.

A fully grown culture on Dubos Tween medium was thoroughly washed and suspended in saline at a concentration of 8×10^6 cells per cc. To 5 cc. of this was added 15 cc. of enzyme solution, containing 500 units (tributyrin) per cc. (4). The mixture was kept at 37° C. and after five days, two weeks, and three weeks, 1 cc. amounts were washed in saline and inoculated on Lowenstein medium. Controls of a similar suspension of H37Rv in heat inactivated enzyme solution were used. The controls show normal growth after six weeks at all periods of testing. Cultures from five days' exposure to the active enzyme showed delayed growth, which was only apparent after 10 weeks as a white, thin, smooth creamy growth, which, one month later (total of three and one-half months' incubation), turned light yellow and spread over the medium. The medium below this growth is very soft. Cultures of suspension exposed a longer time to enzyme action did not give any growth at all.

Microscopically these cultures showed thin long rods mostly disposed in chains. Their standing properties were relative Ziehl fastness and Sudan black B staining as described before. These cultures are, to the best of our knowledge, the first to be made showing tubercle bacilli that stain in Sudan black. At higher concentration or longer than five days' exposure, the enzyme extracts were either bacteriostatic or bacteriocidal.

Study of the Effect of Enzyme Solution on the Neutral Red Cytochemical Reaction of Dubos–Middlebrook

H37Rv cultured for 10 days in Dubos Tween medium, or 21 days in Proskauer-Beck, or six weeks on Lowenstein medium gives a positive neutral red reaction. Identical cultures suspended for three days at 37° C. in enzyme solution of 100 units (tributyrin) (4) per cc., washed, and tested, give a negative neutral red reaction. Bacilli from the BCG strain, grown under identical conditions as the H37Rv above, give a negative neutral red reaction both before and after subjection to enzymes from *Galleria melonella*.

Dubos and Middlebrook (3) claim that the positive neutral red reaction is characteristic of virulent tubercle bacilli as is their cord formation in liquid media. However, the following observation with this test throws some doubt upon this interpretation. When cultures of BCG bacilli have reached a certain age, more than 20 days in Dubos Tween medium, or more than 35 days in Proskauer-Beck medium, and when they have been autoclaved at 105° C. for 20 min., the washed bacilli give a positive neutral red reaction although the reaction was negative before autoclaving. The possible loss of virulence of the neutral red test had therefore to be ascertained by animal experiments.

Study of the Effects of Enzyme Solutions on the Virulence of H37Rv Strain for Guinea Pigs

Ten-day-old cultures of H37Rv bacilli in Dubos Tween 80 medium were centrifuged and twice washed in saline. The discarded liquid was replaced by active (test) and heat inactivated (control) enzyme solution of 100 units (tributyrin) per cc. This concentration of the enzymes was not bacteriostatic

within 20 days of exposure at 37° C. After 1 day, 5 days, 9 days, and 19 days of exposure to the enzyme action, 1 cc. amounts of 1 : 10 dilutions were injected into the groin of each of three guinea pigs. The number of organisms contained in 1 cc. of the 1 : 10 dilution of 10-day-old cultures of H3Rv in Dubos Tween medium corresponds to twice the infecting dose as defined by Middlebrook and Yegian (5). The results of this experiment are given in Table I.

TABLE I

H37Rv, days of exposure to enzyme	Active enzyme					Heat inactivated enzyme				
	Number of animals	Survived 3 months	Generalized T.B.	Local lesions only	Neutral red reaction	Number of animals	Survived 3 months	Generalized T.B.	Local lesions only	Neutral red reaction
1	3	3	3	0	+	3	0	3	0	+
5	3	3	3	0	−	3	0	3	0	+
9	3	3	1	2	−	3	0	3	0	+
12	3	3	0	1	−	3	2	3	0	+
19	3	3	0	0	−	3	2	2	1	(±)

It appears that treatment of virulent bacilli with active enzyme lengthens the survival of infected animals, and the extent of disease is reduced as the period of exposure to enzyme is extended. It is interesting to note that exposure to active enzyme for five days resulted in neutral red negative organisms causing generalized tuberculosis. On this evidence it seems clear that the significance of the neutral red reaction needs further investigation before it can be accepted as a simple test for virulence.

While two animals from the nine days' exposure and one from the 12 days' exposure to active enzyme show a localized lesion (enlarged lymph node on the side of the inoculation) two animals of the 12 days' exposure and all three animals of the 19 days' exposure did not show any lesion whatsoever. In the control group with bacteria treated by heat inactivated enzyme, infectivity was unaltered for 12 days' exposure, and but little reduced in 19 days.

The experiment illustrated the possible progressive loss of virulence of H37Rv bacilli by exposure to the lipidolytic enzymes of *Galleria melonella*.

Study of the Effect of Enzyme Solution on the Streptomycin Sensitivity of H37Rv Bacilli

Ten-day-old cultures of H37Rv bacilli in Dubos Tween medium were centrifuged, twice washed in saline, and exposed in the same way to the same active and inactivated (enzyme control) enzyme solution as in the test of virulence for guinea pigs. After 2, 7, 10, 14, and 20 days of exposure at 37° C., the bacilli were washed, then suspended in as many cc. of saline as the

initial volume of the culture. A volume of 0.05 cc. of this suspension was added to each tube of a set of Dubos Tween medium containing streptomycin in concentrations increasing from 0.025 units per cc. to 10 units per cc. One tube of every set did not contain streptomycin (streptomycin control).

Before enzyme treatment, or treatment by heat inactivated enzyme (enzyme controls) the H37Rv bacilli proved to be sensitive to 0.05 units of streptomycin per cc. At lower concentrations of streptomycin, growth was apparent after 18 days of incubation at 37° C. Bacilli treated by active enzyme show the lag in growth described above and this made it necessary to postpone the reading of the tests to six weeks of incubation at 37° C. Table II shows the effect of the enzymes of *Galleria melonella* on the streptomycin sensitivity of H37Rv bacilli.

TABLE II

Days of exposure to active enzyme (*Galleria mellonella*)	Streptomycin units per cc.								
	10	5	1	0.5	0.25	0.1	0.05	0.025	0
0	−	−	−	−	−	−	−	+	+
2	−	−	−	−	−	−	+	+	+
7	−	−	−	−	−	+	+	+	+
10	−	−	−	−	−	+	+	+	+
14	−	−	+	+	+	+	+	+	+
20	−	+	+	+	+	+	+	+	+

NOTE: + *Indicates growth.*
− *Indicates no growth after six weeks' incubation.*

After two days' exposure to the enzyme at 37° C., there is already a slight decrease in the sensitivity of the bacilli to streptomycin. After 10 days' exposure, the resistance has increased to 0.1 unit streptomycin per cubic centimeter, and after 20 days to five units streptomycin per cubic centimeter. This gradual loss of sensitivity by enzyme treatment is much less than the loss suffered by multiplying bacilli treated by streptomycin. According to Middlebrook and Yegian (5) "resting" bacilli exposed to streptomycin do not acquire streptomycin resistance yet treatment of "resting" bacilli with *Galleria* enzyme results in streptomycin resistance.

Summary

The lipidolytic enzymes of larvae of *Galleria melonella* produce changes in the morphology, the staining properties, the cytochemical reactions with certain dyes, the cultural properties, the virulence, and the streptomycin sensitivity of *Mycobacterium tuberculosis*, strain H37Rv.

Enzyme treated bacilli appear thinner and longer than controls. Instead of the typical cords observed in control cultures, the bacilli are single, or

arranged in chains. They suffer a relative loss of acid fastness, both in the Ziehl-Neelsen and the auramine O stainings. Suspended in enzyme solution or cultured from these suspensions they retain Sudan black B after decolorization by acetone. The affinity of enzyme treated bacilli for Sudan black B dye is demonstrated in a macroscopic test. On the contrary, the affinity of the virulent H37Rv strain for neutral red, as shown in the cytochemical reaction of Dubos and Middlebrook, is lost after enzyme treatment.

According to the concentration of the enzyme used and the length of time of exposure, the bacilli are delayed or arrested in growth. The delayed growth is smooth and spreading and very different to the rough colonies of untreated H37Rv bacilli.

Virulent bacilli exposed to enzyme solution of below bacteriostatic concentration progressively lose their virulence for guinea pigs, until inoculations of 2 LD no longer produce any tuberculous lesions.

Tubercle bacilli thus modified by enzyme treatment show an increase in their streptomycin resistance.

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